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THE IMPORTANCE OF APOPTOSIS OF *Plasmodium*-INFECTED CELLS IN THE GENERATION OF IMMUNITY AGAINST MALARIA INFECTION

Catarina de Almeida Marques

MESTRADO EM MICROBIOLOGIA APLICADA

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ABSTRACT

Despite the efforts towards eradication, malaria remains the most deadly parasitic and vector-borne disease, urging for the development of an effective antimalarial vaccine. Recently, a renewed interest in *Plasmodium* attenuated whole-organism vaccine strategies has emerged, long acknowledged to experimentally induce full and sterile immunity against malaria in mice, monkeys and humans. This approach involves the use of non-replicating but metabolic active sporozoites, either attenuated by exposition to radiation, the Radiation-Attenuated Sporozoites (RAS), or by genetic modification, the Genetically-Attenuated Sporozoites (GAS). These attenuated parasites are able to infect hepatocytes *in vivo* as normal sporozoites, but are unable to fully develop in the liver. This arrest in development has been associated with their apparent failure to prevent host cell apoptosis, which leads to the formation of apoptotic bodies filled with parasite antigens. Several pieces of evidence have suggested that these apoptotic bodies may provide an optimal source of antigens for dendritic cells cross-presentation, and therefore, immune response activation. The aim of this project was to assess whether apoptosis of RAS or GAS infected hepatocytes is involved in the induced protective immunity response against subsequent parasite challenges. Results obtained through the immunization of C57BL/6 background caspase-3 deficient mice with *P. berghei* RAS or *p36p*$^{-}$ GAS revealed that these mice are only partially protected against further infection. These suggest that at least to some extent, caspase-3 mediated apoptosis of infected hepatocytes may be important in generation of immunity by attenuated parasites.

Key words: Malaria, Attenuated Parasites, Host Cell Apoptosis, Immunity.

RESUMO

Em pleno século XXI, e apesar de todas as medidas de controlo implementadas ao longo dos anos, a malária continua a ser uma das doenças mais mortíferas a assolar a humanidade. Só no ano de 2008, a malária foi responsável por 243 milhões de casos de doença e quase um milhão de mortes, maioritariamente no continente Africano.

A malária, ou paludismo, é causada por parasitas pertencentes ao género *Plasmodium*, um grupo de protozoários capazes de infectar grupos diversificados de animais vertebrados como mamíferos, aves e répteis. Em humanos, a malária é causada maioritariamente por quatro espécies, *P. falciparum*, *P. vivax*, *P. ovale* e *P. malariae*, e é transmitida através da picada de mosquitos fêmea infectados do género *Anopheles*. Uma vez inoculado no hospedeiro mamífero, o parasita, sob a forma de esporozoíto, inicia a fase assexuada do seu ciclo de vida, desenvolvendo-se inicialmente assintomaticamente no fígado, onde milhares de merozoítos, a forma do parasita capaz de infectar eritrócitos, são originados. Uma vez libertados na corrente sanguínea, os merozoítos rapidamente infectam eritrócitos, dando origem a mais merozoítos, e iniciando a fase eritrocitária do seu ciclo de vida e sintomática da doença. Contudo, ao fim de alguns ciclos de multiplicação em eritrócitos, alguns merozoítos acabam por gerar gametócitos, as formas sexuais do parasita, que são ingeridas pelo mosquito aquando da sua refeição de sangue. No mosquito, os gametócitos dão início à fase sexuada do ciclo de vida do parasita, que culmina com a formação de esporozoítos capazes de infectar novos hospedeiros mamíferos.

Durante décadas, muitos esforços têm sido empregues com o intuito de erradicar a malária. Contudo, estes têm-se revelado infrutíferos, e tanto parasitas como mosquitos resistentes às medidas de controlo empregues têm emergido nos últimos anos. Perante este cenário, tem-se sugerido que o desenvolvimento de terapias capazes de bloquear a transmissão do parasita do Homem para o mosquito, nomeadamente o de uma vacina eficaz contra a malária, é urgentemente necessário e crucial para se alcançar a erradicação da doença.

Há muito que se tenta desenvolver uma vacina anti-malárica eficaz. No entanto, tal tem-se revelado um dos maiores desafios da medicina, principalmente devido à extraordinária complexidade do ciclo de vida do parasita, durante o qual este se desenvolve e diferencia em formas que apresentam uma morfologia, metabolismo e conteúdo antigénico completamente distintos consoante a fase em que o parasita se encontra. A fase hepática constitui um alvo ideal para o desenvolvimento de uma vacina, uma vez que o desenvolvimento de uma vacina pré-eritrocitária eficaz teria simultaneamente capacidade profiláctica e de bloqueio da transmissão: a eliminação das formas pré-eritrocitárias do parasita (o esporozoíto e a forma exo-eritrocitária em desenvolvimento – EEF - dentro dos hepatócitos), permitiria impedir o aparecimento dos sintomas da infecção e, consequentemente, a transmissão do parasita ao mosquito. Contudo, uma vacina pré-eritrocitária tem que ser 100% eficaz em eliminar o parasita: basta que um esporozoíto se desenvolva completamente no fígado para que ocorra doença e, consequentemente, transmissão.

Vários estudos demonstram que a administração de esporozoítos atenuados mas metabolicamente activos é capaz de induzir uma resposta imune eficiente de longa duração em ratinhos, macacos e humanos, contra futuras infecções por esporozoítos. De facto, a desilusão relativa à eficácia de vacinas baseadas apenas em partes recombinantes de antigénios do parasita,

tem despertado um grande interesse para o desenvolvimento de uma vacina usando esporozoítos vivos atenuados que, em última análise, constitui uma vacina multi-antigénica e em tudo semelhante ao parasita infeccioso.

Os esporozoítos podem ser eficientemente atenuados através da sua exposição a uma determinada dose específica de radiação ionizante (X ou γ) (*radiation attenuated sporozoites* - RAS), ou da sua manipulação genética, em que genes essenciais para o desenvolvimento das formas pré-eritrocitárias do parasita são danificados (*genetically attenuated sporozoites* - GAS). Em ambas as estratégias de atenuação, os esporozoítos atenuados mantêm a sua capacidade de invadir hepatócitos, tal como o parasita selvagem, mas demonstram uma grave deficiência no seu desenvolvimento uma vez dentro da célula hospedeira, que os impede de produzir merozoítos e assim, causar doença. Esta deficiência em completar o seu desenvolvimento dentro do hepatócito tem sido associada com a incapacidade que os parasitas atenuados têm de inibir a apoptose da célula hospedeira.

Ao longo da evolução, os microrganismos intracelulares desenvolveram mecanismos que lhes permitem manipular os processos biológicos da célula hospedeira para seu benefício. Um dos processos que mais pressão selectiva tem imposto aos microrganismos intracelulares tem sido a morte das células hospedeiras por apoptose. Apesar de a apoptose poder ser desencadeada através de diversos estímulos e vias de sinalização, a via extrínseca, accionada por ligandos extracelulares, e a via intrínseca, desencadeada por sinais internos como o stress, são as mais comuns em células de mamíferos. Embora sejam despoletadas por diferentes estímulos, as duas vias apoptóticas convergem na activação de efectores comuns, as proteínas caspase, nomeadamente a da caspase-3, que aquando da sua activação, leva em última instância à morte celular e à resultante formação de corpos apoptóticos. Tal como em outros microrganismos intracelulares, tem sido demonstrado que os estádios pré-eritrocitários de *Plasmodium* possuem a capacidade de inibir a apoptose dos hepatócitos infectados, tanto aquando do processo de invasão, como durante os estádios iniciais e tardios do seu desenvolvimento. Aparentemente, a protecção da célula hospedeira à apoptose durante o desenvolvimento do parasita requer que este se mantenha vivo, e possivelmente, em processo de desenvolvimento. Várias observações sugerem que os parasitas atenuados, tanto RAS como GAS, são incapazes de evitar que a célula infectada entre em processo de apoptose e que, de facto, a imunidade gerada aquando da imunização com os parasitas atenuados resulta da sua incapacidade em proteger a célula hospedeira de entrar em apoptose aquando da infecção.

Quando a célula infectada sofre apoptose, formam-se corpos apoptóticos carregados com antigénios celulares e do parasita. Estes podem depois ser fagocitados por células apresentadoras de antigénios, tais como macrófagos e células dendríticas. Posteriormente, estas células podem fazer a apresentação destes antigénios ao sistema imunitário, desencadeando respostas imunes específicas contra o parasita. Vários estudos têm associado as células dendríticas com o estabelecimento da imunidade protectora induzida pelos parasitas atenuados, apontando-as como o veículo entre as células infectadas em apoptose e o sistema imunitário. De facto, as células dendríticas encontram-se associadas com a apresentação de antigénios exógenos através de moléculas de histocompatibilidade de classe I a células T $CD8^+$, os principais elementos da resposta imune celular gerada pelos parasitas atenuados.

O presente trabalho pretende testar a hipótese de que a apoptose das células infectadas com os parasitas atenuados se encontra intimamente relacionada com o estabelecimento bem sucedido da resposta imune gerada por estes parasitas contra posteriores infecções por esporozoítos infecciosos.

Recorrendo a ratinhos da linhagem C57BL/6 deficientes em caspase-3 (Casp3KO), um elemento-chave em diversas vias da apoptose, vários ensaios de imunização com parasitas *P. berghei* ANKA atenuados, quer por a radiação γ (*Pb*-RAS), ou por manipulação genética (*Pb*p36p$^-$), foram realizados. Em cada ensaio, a posterior infecção foi efectuada com esporozoítos *P. berghei* ANKA que expressam constitutivamente a proteína de fluorescência verde (*Pb*GFP) ou luciferase (*Pb*Luci). Estes parasitas permitem, respectivamente através da análise por PCR quantitativo em tempo real (qRT-PCR) ou imagiologia *in vivo* em tempo real, inferir o nível de infecção no fígado e assim, a eficiência do regime de imunização. Contudo, o estabelecimento de uma resposta imunitária completamente eficiente contra a infecção foi apenas irrefutavelmente depreendida através da vigilância e seguimento do aparecimento de parasitas no sangue dos animais. Estes ensaios permitiram-nos verificar que os ratinhos Casp3KO encontram-se apenas parcialmente protegidos contra a infecção com esporozoítos infecciosos, sugerindo que as vias apoptóticas dependentes de caspase-3 se encontram possivelmente envolvidas no estabelecimento da imunidade induzida pelos parasitas atenuados.

De modo a excluir a possibilidade de que a imunidade parcial observada nos ratinhos Casp3KO se deve a uma deficiência ao nível do sistema imunitário destes animais, uma vez que se pensa dever à diminuição do nível de apoptose das células infectadas no fígado, a medula óssea de ratinhos Casp3KO foi substituída pela de ratinhos C57BL/6 normais, e vice-versa. Os ratinhos quimera resultantes foram imunizados com *Pb*-RAS, e posteriormente infectados com *Pb*Luci. Infelizmente, este ensaio não nos permitiu retirar quaisquer conclusões concretas, apesar de sugerir que o fenótipo observado nos ratinhos Casp3KO imunizados se deve à sua maior resistência à apoptose nas células hepáticas infectadas com os parasitas atenuados.

Através de uma sonda fluorescente que permite detectar níveis de apoptose *in vivo*, foi-nos possível inferir que há um aumento do nível de apoptose no fígado de ratinhos C57BL/6 infectados com *Pb*RAS, que é aparentemente superior ao observado em ratinhos Casp3KO infectados com os mesmos parasitas, e ratinhos C57BL/6 infectados com parasitas infecciosos. Estes resultados permitem-nos de certo modo confirmar as observações de que os parasitas atenuados por radiação são incapazes de proteger a célula hospedeira de sofrer apoptose.

Em conjunto, os resultados aqui apresentados aparentam suportar a hipótese proposta, de que a apoptose das células infectadas com parasitas atenuados é relevante para o estabelecimento da resposta imune efectiva induzida por estes. Perante este panorama, planeámos construir um parasita *P. berghei* ANKA transgénico que expressa e exporta para o citoplasma das células hospedeiras caspase-2, um factor pró-apoptótico, com o intuito de induzir a apoptose das células infectadas e inferir se a infecção de ratinhos com este parasita é capaz de induzir uma resposta imune eficaz contra infecções posteriores com esporozoítos infecciosos *P. berghei* ANKA.

Palavras-chave: Malária, Parasitas Atenuados, Apoptose da célula hospedeira, Imunidade.

MOST USED ABBREVIATIONS

BS – Blocking Solution.

Casp3KO mice - C57BL/6 background caspase-3 deficient mice.

$CD8^+$ T_{CM} – central memory $CD8^+$ T cells.

$CD8^+$ T_{EM} – effector memory $CD8^+$ T cells.

ChimC57BL/6 – C57BL/6 mice reconstituted with Casp3KO mice bone marrow.

ChimCasp3KO - Casp3KO mice reconstituted with C57BL/6 mice bone marrow.

CM – cerebral malaria.

CSP – circumsporozoite protein.

DC – Dendritic Cell.

EEF – Exoerythrocytic form.

ELISA – enzyme-linked immunosorbent assay.

ELISPOT – enzyme-linked immunosorbent spot assay.

GAS – Genetically Attenuated Sporozoites.

i.p. – Intra-peritoneal.

i.v. – intravenously.

ImmC57BL/6 – group of C57BL/6 mice that were immunized, either with *Pb*RAS or *PbP36p*$^-$ sporozoites, and subsequently infected (challenged) with WT *Pb*GFP sporozoites.

ImmCasp3KO – group of Casp3KO mice that were immunized, either with *Pb*RAS or *PbP36p*$^-$ sporozoites, and subsequently infected (challenged) with WT *Pb*GFP sporozoites.

InfC57BL/6 – group of C57BL/6 mice that were only infected.

InfCasp3KO – group of Casp3KO mice that were only infected.

Inf-IrrC57BL/6 – C57BL/6 mice infected with *Pb*GFP-RAS.

Inf-IrrCasp3KO – Casp3KO mice infected with *Pb*GFP-RAS.

N.I.C57BL/6 – non-infected C57BL/6 mice.

NIrr – Non-irradiated.

p.i. – post infection.

***Pb*GFP** – green fluorescent protein (GFP) – expressing *P. berghei* ANKA.

***Pb*GFP-Irr Hosp** – *Pb*GFP parasites that were exposed to a γ-radiation dose of 16 Krad in a Compagnie Oris Industrie IBL 437C irradiator.

***Pb*GFP-Irr IMM** – *Pb*GFP parasites that were exposed to a γ-radiation dose of 16 Krad in a MDS Gamma Cell 3000 *Elan* irradiator.

***Pb*GFP-RAS** – Radiation attenuated *Pb*GFP sporozoites.

***Pb*Luci** – luciferase – expressing *P. berghei* ANKA.

***Pbp36p*$^-$** - P36p deficient GFP – expressing *Plasmodium berghei* ANKA.

***Pb*RAS –** *Plasmodium berghei* radiation attenuated sporozoites.

PV and **PVM** – parasitophorous vacuole and parasitophorous vacuole membrane.

qRT-PCR - Quantitative Real-Time Reverse Transcription PCR.

RAS – Radiation Attenuated Sporozoites.

RBM – Roll Back Malaria partnership.

RT – Room temperature.

WHO – World Health Organization.

WT – Wild Type.

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INTRODUCTION

MALARIA

Malaria is a disease that, despite all the efforts towards its eradication, and all the scientific breakthroughs, remains, in the 21st century, the world's most deadly and morbid parasitic and vector-borne infectious disease. Presently, malaria is a life-threatening risk for 3,3 billion people over 109 countries, in the African, Asian and South American continents (**Figure 1.**). In 2008 alone, it was responsible for as many as 243 million cases of disease and 863.000 deaths, 85% of the losses being African children under the age of 5 (WHO, 2009). Moreover, malaria has an overwhelming impact on the affected countries' economy, being annually accountable for a decrease in the gross domestic product (GDP) of as much as 1.3% in countries with high levels of transmission, and has consequently lead to economical and social disparities between countries with and without malaria (WHO, 2010).

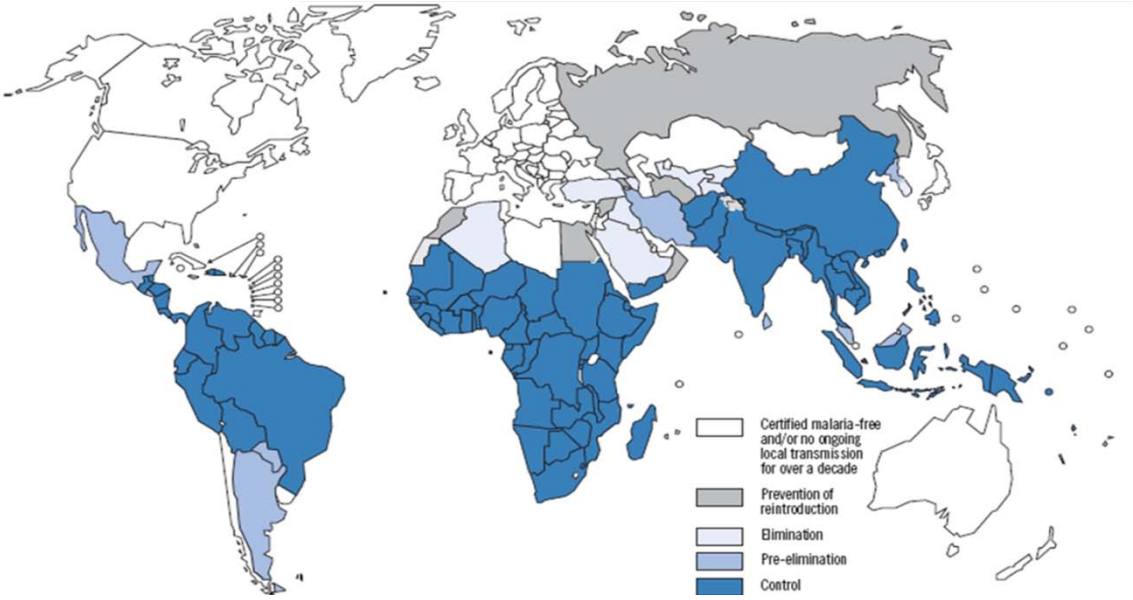


Figure 1. Global map of malaria-free countries and malaria-endemic countries in phases of control, pre-elimination, elimination and prevention of reintroduction by the end of 2008 (WHO, 2009).

Malaria is caused by protozoan parasites from the *Plasmodium* genus, and a member of the Apicomplexa phylum, to which other human pathogens, such as *Toxoplasma gondii* and *Cryptosporidium parvum*, also belong (Ejigiri and Sinnis, 2009). By 2008 there were 199 *Plasmodium* described species that, as a group, are able to infect a broad range of vertebrate hosts such as mammals, birds, and squamat reptiles (Martinsen *et al*., 2008), although each *Plasmodium* species has a remarkably restricted host range, and typically only infects few closely related vertebrate species (Matuschewski, 2006). In humans, malaria is mainly caused by four *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, although a fifth species, *P. knowlesi*, a natural parasite of macaque monkeys in south-east Asia, has been recently described to have occasionally infected and caused disease in humans (WHO, 2010). Together with *P.* vivax, *P. falciparum*, which is responsible for most of the severe and deadly malaria cases, accounts for the vast majority (over 93%) of the clinical cases notified each year (WHO, 2009), while *P. ovale* and *P. malariae* contribute to a lesser number of malaria infections (RBM, 2008). Malaria is transmitted to the vertebrate host by the bite of infected female mosquitoes, members of genera that comprise the most ubiquitous and

abundant arthropod populations in most parts of the world: *Anopheles* in the case of mammalian malaria, and both *Culex* or *Aedes* in the case of avian malaria (Matuschewski, 2006). In fact, the use of such vectors constitutes one of the main reasons for malaria's success.

Plasmodium LIFE CYCLE

Plasmodium parasites possess a complex life cycle, divided into two obligatory hosts where asexual, in the vertebrate, and sexual, in the mosquito, stages take place. During both, the parasite follows a developmental programme where it changes from extracellular motile invasive stages, to intracellular replicative ones (Matuschewski, 2006).

While taking its blood meal from a mammal host, the infected female *Anopheles* mosquito deposits sporozoites, a parasite infective form that is unable to infect erythrocytes, into the host avascular dermal tissue (Amino *et al*., 2006) (**Figure 2. A**). Here, sporozoites persist for some time, 1-3 hours (Amino *et al*., 2006; Yamaushi *et al.*, 2007), apparently moving in a random fashion by gliding motility, an active movement pattern characteristic of *Plasmodium* spp. This is accompanied by active migration through the host cells with membrane disruption, and is required for sporozoite exit from the dermis. Migration, by traversing cells, allows the sporozoites to penetrate cell barriers, to escape destruction by phagocytic cells in the dermis, and to reach successfully the bloodstream (Amino *et al.*, 2008). However, only a proportion of the initially inoculated sporozoites leaves the dermis, penetrates a blood vessel, and is carried within the bloodstream. Some sporozoites remain in the dermis, where they are probably eliminated by recruited phagocytes, while others penetrate a lymphatic vessel and reach the lymph node. Here, sporozoites are either phagocytosed by dendritic cells (DCs) or partially develop into early small exoerythrocytic forms (EEFs), before being degraded (Amino *et al.*, 2006). Once in the bloodstream, sporozoites rapidly home to the liver, starting an obligatory and clinically silent liver, or pre-erythrocytic, stage of infection that, in humans, can last for 6 to 40 days, depending on the *Plasmodium* species (Murray *et al.,* 2005). In the liver, sporozoites glide freely along the sinusoidal epithelium until they get sequestered. Here, they invade Kupffer cells, the liver's resident macrophages, and gain access to the liver parenchyma (Frevert *et al*., 2005) (**Figure 2. B**). Sporozoites then migrate through several hepatocytes, before productively invading a single final one with the formation of a parasitophorous vacuole (PV) (Mota *et al.*, 2001) (**Figure 2. C**). Afterwards, each invasive sporozoite dedifferentiates and develops into an EEF that grows and increases dramatically in volume without annihilating the host cell (reviewed in Vaughan *et al*., 2008) (**Figure 2. D**). During its growth, the EEF multiplies by schizogony (a process in which karyokinesis is temporally separated from cytokinesis, and a hallmark of *Plasmodium* spp. replication stages; Aly *et al*., 2009) into thousands of merozoites, the parasite's next infective form capable to infect erythrocytes, that stay enclosed within the PV membrane (PVM) (Sturm *et al*., 2006). When EEF development is completed, merozoites are released from the PVM freely in the host cell cytoplasm, after which merozoite-filled vesicles from host cell membrane origin, the merosomes, are formed and released into the liver sinusoid (Sturm *et al*., 2006) (**Figure 2. E**).

Once in the liver sinusoids, the merosomes enter the bloodstream and accumulate in the lung's microvasculature, where they finally break up releasing merozoites into the bloodstream (Baer *et al*., 2007; Strum *et al.*, 2006) (**Figure 2. F**), and starting the symptomatic blood, or erythrocytic, stage of infection. Merozoites then rapidly attach to the erythrocytes, further penetrating them with the

formation of a PV. Subsequently, the invasive merozoite converts into a ring stage form, which develops into a trophozoite stage. The trophozoite then starts to multiply by schizogony, transforming itself into an erythrocyte schizont stage, which ultimately originates newly formed merozoites. When fully developed, merozoites egress from the erythrocyte, by PV and erythrocyte membrane rupture, after which they rapidly infect other erythrocytes, initiating a new erythrocytic cycle (reviewed in Silvie *et al*., 2008) (**Figure 2. F**). It is when the erythrocytes release merozoites that the typical pattern of malaria symptoms appears: the paroxysms, consisting of chills, high fever and malarial rigors (Murray *et al.,* 2005). These usually appear periodically, the time interval depending on the length of the *Plasmodium* species erythrocytic cycle (in humans can be of 24-72 hours), and may remain relatively mild, or progress to severe attacks with hours of sweating, chills, persistent high fever and exhaustion (Murray *et al.,* 2005). If not treated, *P. falciparum* malaria evolves to a severe, and maybe fatal, disease with involvement of the brain, cerebral malaria, due to the accumulation of parasitized cells and cellular debris. After several erythrocytic cycles, some merozoites give origin to the sexual stages, the male and female gametocytes (**Figure 2. G**).

When a mosquito takes its blood meal from an infected individual, gametocytes are ingested, starting the sexual part of the parasite's life cycle (**Figure 2. H**). Here, the midgut lumen environment triggers gametocytes to rapidly undergo gametogenesis (Bilker *et al.*, 1997; Bilker *et al.*, 1998), originating the male and female gametes that subsequently fuse to form the zygote, the only diploid form during the parasite's life cycle. Soon after its formation, the zygote endures meiosis and genetic recombination, converting itself into an ookinete, a motile form of the parasite. Thereafter, the ookinete breaches the peritrophic matrix that encloses the blood meal and penetrates the mosquito midgut epithelium, where it traverses several epithelial cells. Subsequently, it exits the epithelium, and initiates its transformation into a sessile oocyst, the only extracellular developmental stage of the

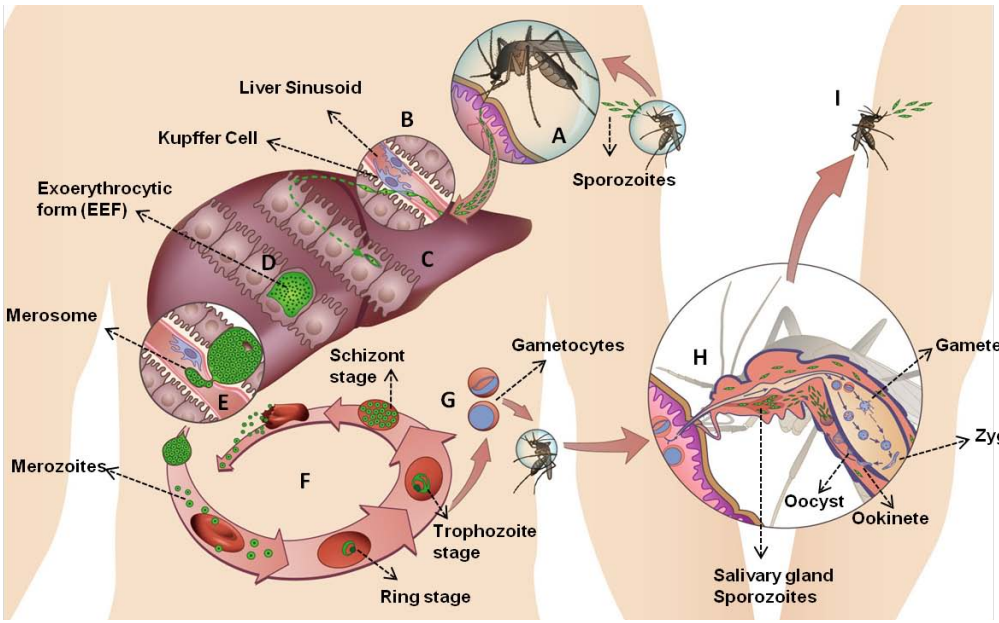


Figure 2. *Plasmodium* spp. life cycle. Detailed description in the text. **(A)** The infected mosquito deposits sporozoites in the host's skin where they gain access to a blood vessel. **(B)** In the liver sinusoids, sporozoites invade Kupffer cells and get access to the liver parenchyma. **(C)** Sporozoites migrate through several hepatocytes, invading a final one with formation of a PV. **(D)** Sporozoites then develop into EEFs, giving rise to thousands of merozoites. **(E)** Merosomes are release from the infected hepatocyte into the sinusoid lumen. **(F)** Erythrocytic cycle. **(G)** Some merozoites originate the male and female gametocytes. **(H)** During a blood meal, the mosquito uptakes the gametocytes, starting the parasite's sexual part of the life cycle that ends with the accumulation of thousands of sporozoites in the salivary glands. **(I)** The mosquito takes a blood meal, and the cycle restarts. Adapted from *Plasmodium* life cycle from Malaria Unit of IMM.

parasite (reviewed in Aly *et al*., 2009). Afterwards, the oocyst develops, undergoing several mitotic divisions to originate sporoblasts, from which sporozoites start budding. When fully developed, thousands of mature sporozoites actively egress the oocyst and enter the hemocoel, where are carried through all mosquito tissues by the circulatory system, the hemolymph. While passing through the salivary glands, sporozoites adhere to the basal lamina, breach it, and invade the salivary gland secretory acinar cells, getting access to the salivary duct, where they accumulate and finish their maturation (reviewed in Aly *et al*., 2009). When the mosquito takes its next blood meal, sporozoites are injected into the mammalian host, starting the asexual part of the parasite's life cycle (**Figure 2. I**).

MALARIA ERADICATION EFFORTS AND CONTROL MEASURES

Throughout time, many have been the efforts to control, eliminate and, ultimately, eradicate malaria. However, these have not been completely successful, although malaria elimination was effectively achieved in Europe, North America and some countries in the North of Africa (**Figure 1.**). In 1959, the World Health Organization (WHO) launched the first attempt to eradicate malaria, the Global Programme for Malaria Eradication, which was based on mosquito control through indoor residual spraying (IRS) with dichloro-diphenyl-trichloroethane (DDT), and treatment of the infected populations with chloroquine (Greenwood, 2009). Sadly, the ambitious programme did not succeed in its main goal, and was abandoned in 1969. Nevertheless, it allowed malaria elimination in over 30 countries, a decrease of clinical burden in several endemic countries outside the African continent, and the removal of malaria risk from over one billion people (Greenwood, 2008; Greenwood, 2009). After the programme was abandoned, for some time no other attempts were made to eradicate malaria, and the efforts were directed to regional control and elimination, as has been done previously to the programme for years, back then, with limited success (Greenwood, 2009). In 2007, the Melinda and Bill Gates Foundation, the WHO, and the Roll Back Malaria Partnership (RBM) joint efforts to accomplish malaria eradication. This time, eradication will be endorsed by promoting national and regional gradual elimination using combinations of tools that are currently available: the artemisinin combination therapies (ACTs), the chemoprophylaxis or intermittent preventive treatment (IPT), IRS, and insecticide–treated bed nets (ITNs) or long-lasting ITNs (LLITNs) (Greenwood, 2008; Greenwood, 2009). It is believed that in many epidemiological situations the correct widespread and combination of these tools will be able to achieve up to 90% reduction in malaria clinical burden regionally. However, it is doubtful that in medium or high transmission areas these will be enough to accomplish malaria elimination and ultimately, eradication (Greenwood and Targett, 2009). Moreover, the emergence of resistance against several antimalarials by the parasite, and against several insecticides by the mosquito, threaten their efficacy. Therefore, it is argued that tools efficient in blocking parasite transmission, such as new gametocytocidal drugs, safer than primaquine, along with an efficient antimalarial vaccine, are essential and crucial for ultimate malaria eradication (reviewed in Greenwood, 2008).

MALARIA VACCINATION STRATEGIES

Since the early 19th century, researchers have struggled to develop an effective antimalarial vaccine, long acknowledged as one of the greatest challenges of medicine (Luke and Hoffman, 2003). The main reason for this fruitless quest relies on the complexity of the parasite's life cycle, comprising three distinct stages, each with a different gene expression pattern and consequently, different morphology, metabolism and antigen content (Vaughan *et al*., 2010). The pre-erythrocytic stages of the parasite, the sporozoite and the developing EEF, constitute attractive targets for vaccine development: they are present in lower numbers than the erythrocytic stages, are completely asymptomatic, and provide a relatively large window of opportunity for an effective immune response to eliminate the parasite (Vaughan *et al*., 2010), given that parasite development in the human liver lasts for several days. Moreover, pre-erythrocytic stages appear to not exhibit substantial antigenic variation or antigenic polymorphisms (Vaughan *et al*., 2010), conferring to a pre-erythrocytic vaccine the potential of being effective against heterologous parasite strains. If completely efficient, a pre-erythrocytic vaccine would promote the elimination of the parasite in the liver, therefore preventing disease onset, and consequently, parasite transmission. In fact, the major challenge for a successful pre-erythrocytic vaccine is that it is an all-or-none vaccination strategy, as it must eliminate every single parasite in the liver because, if even just one is able to fully develop, the parasite will proceed to the blood, cause disease and consequently, be transmitted (Vaughan *et al*., 2010).

The development of an effective pre-erythrocytic vaccine has relied mainly on two strategies, the subunit and the live attenuated whole organism approaches.

THE SUBUNIT VACCINE APPROACH

Most of the efforts to develop an effective vaccine against malaria have focused on the recombinant subunit approach. Several vaccine candidates have emerged through time, but the majority proved to be inefficient in inducing protective immunity in humans and were early abandoned. However, two strong candidates have reached human clinical trials[a], the Sf66 and the RTS,S vaccines, both comprising parts of the *P. falciparum* circumsporozoite protein (CSP), which is only expressed in the pre-erythrocytic stages of the parasite, both in the sporozoite and the EEF (Greenwood and Targett, 2009).

Sf66 was the first vaccine candidate to incorporate peptide sequences derived from both pre- and erythrocytic stages of the parasite life cycle. Sf66 was based on a synthetic peptide unit that, besides comprising the immunodominant unit NANP (N, asparagines; A, alanine; P, proline) of the CSP, also incorporated a fragment of the merozoite surface protein 1 (PfMSP-1, involved in the merozoite initial attachment to the erythrocyte upon infection) (reviewed in Silvie *et al*., 2008), and two uncharacterized peptides of 55 and 35KDa (Snounou and Rénia, 2007). Initially, Sf66 showed strong evidence of conferring protection against *P. falciparum* blood-stage challenge in monkeys and human volunteers. Facing such results, Sf66 was further tested, between 1992 and 1999, in several independent clinical

[a] **Steps in malaria vaccine development. (1)** ***Research and preclinical development*** **–** identification of antigens and creation of the vaccine concept, preclinical evaluation in animal models and development of the manufacturing process. **(2)** ***Phase I Clinical Trials (CT)*** **–** safety profile and immune response preliminary evaluation in naïve and exposed populations. **(3)** ***Phase II CT*** **–** safety and potential side effects monitoring, immune response measurement, efficacy against infection and clinical disease evaluation and determination of the optimum dosage and schedule. **(4)** ***Phase III CT*** **–** further safety and potential side effects monitoring and efficacy evaluation on a large scale. **(5)** ***Submission to regulatory authorities.*** **(6)** ***Introduction in the market.*** **(7)** ***Phase IV CT*** **–** post-marketing safety monitoring, protection duration measurement and vaccine compliance assessment. (adapted from GSK, 2010)

trials in endemic countries. Unfortunately, it showed a reduced efficacy against infection, and was abandoned after being considered of no use in malaria control (Snounou and Rénia, 2007).

The RTS,S vaccine is the most clinically advanced antimalarial vaccine candidate so far. It is based on the hepatitis B surface antigen (HBsAg or S antigen) virus-like particle platform, genetically engineered to harbour a *P. falciparum* CSP construct comprising 19 NANP repeats of the protein central region, known to induce B cell responses, and its entire C-terminal flanking region, known to induce specific T cell responses (Casares *et al*., 2010). To enhance its immunogenicity, the RTS,S construct was formulated in association with several adjuvant systems (AS), shown to induce strong humoral and cellular immune responses. Two formulations, RTS,S/AS02 and RTS,S/AS01 have shown promise results in studies with human volunteers, and were further tested in phase II clinical trials in endemic countries (GSK, 2010). In these clinical trials both formulas were shown to be safe to use as a vaccine in young children, with a strain unspecific efficacy against infection of about 45%, sadly far from ideal. Unexpectedly, since RTS,S was designed to act on pre-erythrocytic stages by only incorporating CSP as an immunogen, RTS,S formulations have also shown to be effective against clinical malaria, with around 44% efficacy, and against severe malaria development, with nearly 49% efficacy (Vekemans *et al*, 2009; Casares *et al*., 2010). Unfortunately, the efficiency of both formulas tended to decline with time. Altogether, RTS,S/AS01 showed a higher efficiency and more favourable safety data than RTS,S/AS02, and was selected for further evaluation in a phase III pre-licensure pivotal efficiency trial in several endemic countries, meaning that, a partially effective antimalarial vaccine will, hopefully, be available in a near future (Vekemans *et al*, 2009). Nonetheless, giving that the RTS,S has an efficacy lower that 50% against infection, it is arguable whether RTS,S will be useful when added to the other control measures in the quest to eradicate malaria (90% with the current tolls compared to 93% when combined with the vaccine). Moreover, the fact that it is only based on one *P. falciparum* protein raises the concern of whether a single protein based vaccine will be adequate to sustain malaria control, especially because it may bear the risk of emergence of resistant parasites (Greenwood and Targett, 2009; Luke and Hoffman, 2003).

THE LIVE ATTENUATED WHOLE ORGANISM VACCINE APPROACH – RAS & GAS

The observation that subunit vaccines fail to provide any significant and long lasting protective immunity has stimulated recent attempts to produce a vaccine based on an alternative approach, in which non-replicating but metabolically active live attenuated whole organisms are used. In fact, attenuated whole organism vaccines represent 75% of the currently licensed anti-viral and anti-bacterial vaccines (Good, 2005), as it is the case of the ones against poliovirus, measles, typhoid fever, and tuberculosis (reviewed in Hoffman *et al*., 2010). Throughout the years, it has been shown that this approach can, at least in theory, be applied to the development of a highly effective antimalarial vaccine.

In 1967, it was demonstrated that immunization of mice with *P. berghei*, a rodent malaria parasite, radiation-attenuated sporozoites (RAS) was able to completely abrogate the onset of blood stage infection after challenge with infectious *P. berghei* sporozoites (100% efficacy) (Nussenzweig e*t al*., 1967). Subsequently, the same level of sterile protection against infection was also induced in monkeys (Collins and Contacos, 1972) and humans (Clyde *et al.*, 1973), immunized with *P. cynomolgi* RAS and *P. falciparum* RAS, respectively. Moreover, the protection conferred by immunizations with

RAS was shown to be long-lasting either in mice, monkeys or humans (Nussenzweig *et al*., 1967; Clyde *et al.*, 1973; Hoffman *et al.*, 2002). Soon, it became evident that the safety and efficacy of the RAS parasites relied on a precise and adequate radiation dose (ionizing radiation, either X- or γ-radiation). The proper irradiation of the sporozoites introduces random mutations and breaks into the DNA, to an extent that allows them to survive and keep their ability to successfully invade hepatocytes, although their capacity to fully develop is completely abrogated, and therefore, infection with these RAS does not cause disease and parasite transmission (**Figure 3. B**) (Vaughan *et al*., 2010). Conversely, under-irradiation of sporozoites allows them to remain infectious and to fully mature in the liver, therefore causing malaria, while over-irradiation completely inactivates or kills the sporozoites, that consequently are not able to infect hepatocytes (Vanderberg *et al*., 1968). In fact, over-irradiated sporozoites fail to confer any significant protection, suggesting that antigens expressed by persistent, partially developed liver stage parasites may be critical for the induction and maintenance of protective immunity, thereby indicating that RAS must invade liver cells and persist as at least partially developed EEFs to induce an immune response (Ballou, 2007). Because RAS

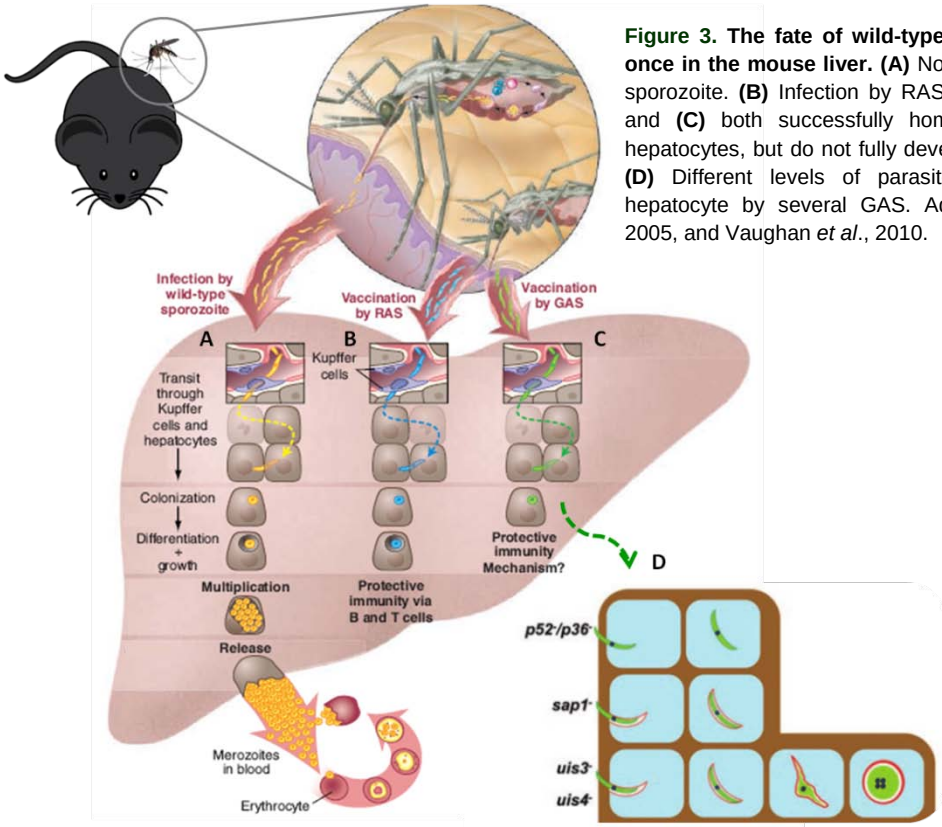


Figure 3. The fate of wild-type and attenuated parasites once in the mouse liver. (A) Normal infection by a wild type sporozoite. **(B)** Infection by RAS. **(C)** Infection by GAS. **(B)** and **(C)** both successfully home to the liver and infect hepatocytes, but do not fully develop to generate merozoites. **(D)** Different levels of parasite development inside the hepatocyte by several GAS. Adapted from Waters *et al*., 2005, and Vaughan *et al*., 2010.

comprehend the entire parasite, they act as a complete multi-antigen vaccine, possessing all the antigens of the infective non-irradiated sporozoites (N-Irr). Therefore, the resulting immune response is quite complex, involving both cellular and humoral immune responses against infected hepatocytes and surface antigens present in the sporozoite surface, like the CSP (reviewed in Ballou, 2007).

Despite all the promising results of obtaining 90-100% efficacy of protection against infection in humans with RAS (Hoffman *et al.*, 2002), for decades it was considered impossible to develop, license and commercialize a live attenuated whole organism vaccine for *P. falciparum* (Ballou, 2007). The main obstacles to its development were mainly associated with the processes of producing, collecting and purifying the sporozoites, which present a high risk of contamination with other potentially

pathogenic transmissible agents, from both human and mosquito origin, as well as with allergenic mosquito proteins. These concerns come from the fact that sporozoite production involves feeding the mosquitoes with gametocyte-infected human blood (always considered to carry an inherent non-zero risk of infectious agents), and further sporozoite collection and purification, involving the removal and disruption of the mosquito's salivary glands. Moreover, the capability of scaling up these processes has also been a great limiting factor, as well as how this type of vaccine would be preserved and taken to the endemic areas (Ballou, 2007). In 2003, Stephen Hoffman, founder of the Sanaria Inc., and co-workers reappraised the potential of an attenuated whole organism vaccine against malaria, as well as the obstacles to produce it, and took the challenge to develop such a vaccine (Luke and Hoffman, 2003). Presently, Sanaria Inc. possesses the world's first licensed facility for manufacturing a live attenuated malaria vaccine, where an entire single coordinated process of producing and preserving the first generation attenuated whole organism vaccine against *P. falciparum* malaria, the Sanaria™ PfSPZ vaccine, has been developed (Hoffman *et al*., 2010). This tightly controlled process has enabled Sanaria Inc. to overcome the risks and limitations of contaminations, improper radiation of the sporozoites, as well as their further preservation and delivery to endemic countries, by cryopreservation, storage and distribution of the RAS in liquid nitrogen vapor phase (LNVP), an electricity-independent platform (Hoffman *et al*., 2010). Currently, the Sanaria™ PfSPZ vaccine, given by intradermal (ID) or subcutaneous inoculation (SC) is being tested in phase I clinical trials, in experimentally challenged malaria naïve volunteers to assess its safety, immunogenicity, adequate dose, and protective efficacy (Vaughan *et al*., 2010). Ultimately, during clinical trials Sanaria™ PfSPZ must demonstrate that it is aseptic (free from contaminating pathogens), pure (free of contaminating mosquito material), non-replicating (arrests during EEF development and is thus unable to cause malaria), and potent (capable of inducing protective immune response), in order to be introduced in the market (Hoffman *et al*., 2010). If it develops favourably, after all these years of research, an attenuated whole organism vaccine against malaria, with a high efficacy of protection, may finally be available in a relatively near future, and become an important tool to achieve malaria eradication.

Although the efforts and breakthroughs accomplished by Sanaria Inc. appear to finally make feasible the development of a live attenuated whole organism vaccine using RAS, there will always be reservations regarding the safety and reproducibility of the radiation process (Ballou, 2007), as it is unacceptable that a vaccine itself may cause disease if it is improperly attenuated. Fortunately, advances in genetic engineering may have allowed researchers to overcome this problem. The availability of genome sequences for a number of *Plasmodium* species, including of *P. falciparum*, the generation of stage-specific gene expression data, and the capacity to genetically manipulate the parasite, has allowed the search for essential genes involved in parasite survival at different stages of its life cycle. Together, these have enabled the informed genetic manipulation of the parasite to produce genetically-attenuated sporozoites (GAS), that can be designed to exhibit distinct biological and antigenic features, therefore allowing the creation of strains that are safe and elicit complete protection, with low dose immunizations (Vaughan *et al*., 2010).

It has been demonstrated that the gain of infectivity by sporozoites (from non-infective sporozoites in the midgut to infective sporozoites inside the salivary glands) is accompanied by an extensive differential upregulated expression of the upregulated in infectious sporozoites (UIS) genes

(Matuschewski *et al.*, 2002). Disruption of several of UIS genes, which the parasite lacks the ability to compensate for their loss, has been done in rodent parasites like *P. berghei* and *P. yoelii*, and allowed researchers to test whether it is feasible to develop an efficient genetically-attenuated antimalarial vaccine. In *P. berghei,* disruption of the *UIS3* and *UIS4* genes, both encoding transmembrane proteins present in the EEF PVM, as well as of the *P36p* gene (also termed *P52*), encoding for a putative glycosylphosphatidylinositol (GPI)-anchored protein, member of the 6-Cys domain protein family, has been successfully achieved by double cross-over recombination gene replacement. The resulting parasites, *uis3*$^{-}$*, uis4*$^{-}$ and *p36p*$^{-}$, have revealed complete arrest in EEF development soon after hepatocyte invasion (**Figure 3. D**), although all three have shown, as expected, to present a phenotype comparable to the one of the wild type (WT) parasite regarding the blood stage and mosquito stage infections, as well as hepatocyte migration and invasion processes (Mueller *et al*., 2005a; Mueller *et al*., 2005b; van Djik *et al*., 2005). However, in the case of the *p36p*$^{-}$ parasites, a consensus regarding the later processes has not been established. In two independent studies where *p36p*$^{-}$ parasites were generated using similar strategies, one reported an increase in migration and a decrease in invasion with PV formation (Ishino *et al*., 2005), while the other described that both processes are comparable to those of the WT parasite, although invasion occurred without the formation of a PV (van Djik *et al*., 2005). Nevertheless, when mice were immunized with *uis3*$^{-}$*, uis4*$^{-}$ or *p36p*$^{-}$ parasites, complete and long-lasting immunity was generated against further challenges with WT sporozoites (Mueller *et al*., 2005a; Mueller *et al*., 2005b; Ishino *et al*., 2005; van Djik *et al*., 2005), demonstrating that rodent model GAS are highly effective vaccines. Unfortunately, when given to mice in high doses, *uis4*$^{-}$ and *p36p*$^{-}$ parasites (from both studies) were reported to cause breakthrough infections, although with a delayed appearance of parasites in the blood (Mueller *et al*., 2005b; Ishino *et al*., 2005; van Djik *et al*., 2005), which may surrender their potential to be used as vaccines. Recently, successful induction of complete long-lasting immunity was also reported to be achieved by disruption of other sporozoite specific gene, the *SAP-1* (sporozoite asparagine-rich protein-1) in *P. yoelii* (Aly *et al*., 2008). The *sap1*$^{-}$ parasites showed a phenotype comparable to the one of the WT regarding blood stage and mosquito infections, as well as migration and invasion of hepatocytes. Resembling what was described for the three aforementioned GAS, *sap1*$^{-}$ parasites showed complete early EEF development arrest (**Figure 3. D**) and, like the *uis3*$^{-}$ parasites, no breakthrough infections occurred, even when extremely high doses of *sap1*$^{-}$ sporozoites were injected into the mice. The *sap1*$^{-}$ parasites were shown to lack the expression of essential genes such as the *UIS3, UIS4* and *P36p* genes, suggesting SAP-1 may be involved in a post-transcriptional mechanism of gene expression control, and that actually, more genes may be also abrogated in this parasite (Aly *et al*., 2008). This lack of several genes expression turns the *sap1*$^{-}$ parasite into an attractive GAS vaccine candidate due to its quasi-multi-loci attenuation.

Altogether, the data obtained with rodent models GAS, especially of *uis3*$^{-}$ and *sap1*$^{-}$ parasites, have shown that safe and highly protective live attenuated malaria parasites can be created by genetic manipulation. Moreover, these studies may allow researchers to transfer the knowledge acquired to the development of a *P. falciparum* GAS, since all the aforementioned genes possess orthologues in other *Plasmodium* species, including *P. falciparum* (Mueller *et al*., 2005a; Mueller *et al*., 2005b; Ishino *et al*., 2005; Aly *et al*., 2008).

Recently, to assess the potential to create a GAS vaccine for human malaria, the *P36p* and the *P36*, a non-UIS gene encoding a putative secreted protein member of the 6-Cys domain protein family, genes were simultaneously disrupted in *P. falciparum* by double cross-over recombination gene replacement, to avoid genetic reversion and reconstitution of the WT locus (VanBuskirk *et al.* 2009). The resulting *p36p⁻/p36⁻* parasites presented a phenotype comparable to the one of the WT parasite regarding the blood stage and mosquito stage infections, as well as hepatocyte migration and invasion processes. Moreover, *P. falciparum p36p⁻/p36⁻* parasites exhibited a profound EEF development arrest, and were not able to survive beyond the third day post infection (p.i.), both *in vitro* (using a human hepatic cell line) and *in vivo* (using chimeric mice carrying human hepatocyte transplant), and therefore, were unable to persist in a growth-arrested state (VanBuskirk *et al.* 2009). These results were in accordance with the ones obtained in the *P. yoelii* model in which *P36p* and the *P36* orthologues were simultaneously disrupted. The *P. yoelii p36p⁻/p36⁻* parasite showed complete arrest of the EEF development after invasion without PV formation (**Figure 3. D**) and no breakthrough infections were reported. Besides, complete immunization against further challenges with WT sporozoites was achieved (Labaied *et al.*2007). Facing such a strong phenotype of EEF development arrest, as well as the promising results obtained using a rodent model, the *P. falciparum p36p⁻/p36⁻* parasite has been selected to advance into phase I clinical trials in human volunteers (Vaughan *et al*., 2010).

Altogether, these results, along with the technologies developed by Sanaria Inc., which can be adapted to GAS, indicate that it is feasible, in a somewhat near future, to generate a safe and efficient *P. falciparum* GAS vaccine that offers the advantages of genetic homogeneity, standardization, batch-to-batch consistency and testable identity over the RAS, and even, the subunit candidates.

LIFE AS AN INTRACELLULAR PATHOGEN – MODULATION OF HOST CELL APOPTOSIS

Attenuated *Plasmodium* parasites, both RAS and GAS, are unable to fully develop inside the infected hepatocyte, arresting their development at early EEF stages. Several pieces of evidence have suggested that this may be due to their inability to prevent host cell apoptosis during development inside the hepatocyte.

By living and developing within a host cell, intracellular pathogens are protected from immediate attacks by mechanisms of the host immune system. To achieve a successful intracellular life style, these pathogens have developed powerful methods to manipulate host cell functions in their own advantage, such as those involved in normal cell proliferation, cellular development and, specially, cell death by apoptosis, which is activated upon external or internal stimuli (van de Sand *et al*., 2005). Apoptosis is an active, highly specific and regulated, physiological process that plays an important role in tissue development and homeostasis, regulation and termination of immune responses and, importantly, in the removal of damaged or infected cells (reviewed in Carmen and Sinai, 2007).

There are two main pathways leading to apoptosis in mammalian cells, the intrinsic and the extrinsic pathways, both converging on common effectors, the caspases (cysteine proteases that cleave after aspartate residues). Briefly, the extrinsic pathway is triggered by the activation of a death receptor on the cell plasma membrane surface (e.g. FasL) following the binding of an external ligand (e.g. Fas). This leads to the formation of the death-inducing signalling complex (DISC) that activates the initiator caspase, caspase 8 that further activates an effector one, the caspase 3. Once activated,

caspase 3 ultimately leads to cell death, by being responsible for multiple proteins activation. Meanwhile, the intrinsic pathway is initiated by internal signals like DNA damage or stress, and relies on the release of cytochrome c from the mitochondria. Once in the cytosol, cytochrome c interacts with the apoptosis activating factor 1 (Apaf-1) that oligomerizes to form an apoptosome, which in turn, recruits and promotes the activation of the initiator caspase 9, further activation of caspase 3 and, consequently, cell death (reviewed in Carmen and Sinai, 2007; James and Green, 2004). Both pathways are tightly regulated by multiple inhibitory or pro-survival mechanisms, such as the pro-survival responses mediated by the phosphatidylinositol 3-kinase (PI3-K) pathway, known to interfere and inhibit apoptosis (reviewed in Carmen and Sinai, 2007).

Although no inflammatory response is elicited when infected cells undergo apoptosis, phagocytes are attracted to the site, where they efficiently recognize and eliminate the apoptotic cell harbouring the intracellular pathogen. Throughout evolution, host cell apoptosis has therefore placed a strong selective pressure on intracellular pathogens, either virus, bacteria and parasites, to inhibit the host cell apoptotic machinery (Heussler *et al.*, 2001). Among protozoans, *Toxoplasma gondii*, *Theileria* spp., *Trypanosoma cruzi* and *Leishmania* spp. are known to confer resistance to host cell apoptosis, and *Plasmodium* spp. is not an exception (reviewed in Carmen and Sinai, 2007).

Once in the liver parenchyma, sporozoites traverse several hepatocytes before productively invading a final one with the formation of a PV (**Figure 1. C**). Upon traversal, wounded hepatocytes secrete hepatocyte growth factor (HGF), probably as a result of transient loss of their membrane integrity. HGF then binds to its tyrosine kinase receptor c-mesenchymal-epithelial transition factor (c-MET) on the surface of neighbour hepatocytes (Carrolo *et al*., 2003), leading to the activation of the HGF/c-MET signalling. This then mediates the coordinated execution of multiple cellular processes that both render the hepatocytes more susceptible for infection (Carrolo *et al*., 2003) and lead to the activation of the pro-survival PI3-K pathway (Leirião *et al*., 2005a). Activation of the PI3-K pathway by HGF/c-MET signalling has been shown, both *in vitro* and *in vivo*, to prevent host cell apoptosis during the initial phases of hepatocyte infection (Leirião *et al*., 2005a). However, this has not been observed at later stages of EEF development, since HGF/c-MET signalling requirement during *Plasmodium* infection of hepatocytes occurs at the early stages of establishment and development of sporozoites within the host cell (Carrolo *et al*., 2003). Nevertheless, prevention of apoptosis during infection mediated by the PI3-K pathway may also be induced by other signalling pathways, or even be mediated by other pathways. This is supported by the report that sporozoite microneme protein essential for cell traversal (SPECT) disrupted *P. berghei* parasites, which lack the ability to migrate through hepatocytes, and therefore, to induce HGF secretion, are able to infect hepatocytes *in vitro* comparably to the WT parasites (Ishinho *et al*., 2004). At later phases of EEF development, the parasite grows and develops far beyond the normal size of the host cell (**Figure 1. D**), exerting an important stress factor on the infected hepatocyte. However, infected hepatocytes were reported to not exhibit signs of cell death and to be protected against apoptosis induced by several stimuli, both *in vitro* and *in vivo* (van de Sand *et al*., 2005). Actually, resistance to apoptosis seems to become more pronounced during the EEF development, and to be independent from the PI3-K pathway. It is more likely that at later stages of hepatocyte infection the parasite blocks potential pro-apoptotic factors,

most probably by secretion of parasite molecules, rather than stimulating host cell survival pathways (van de Sand *et al*., 2005). In the meantime, these mechanisms remain to be elucidated.

Apparently, host cell protection from apoptosis requires that the parasite remains alive. There are indications that *P. berghei* RAS and *P. berghei p36p*$^-$ infected hepatocytes show evidence of apoptosis, like caspase-3 activation, shortly after invasion and early developmental phases. This suggests that these attenuated parasites, which are deficient in completing their development within the hepatocyte, fail to protect the host cell from entering in the apoptotic process (Leirião *et al*., 2005b; van Dijk *et al*., 2005). Interestingly, at the same time point, *p36p*$^-$ parasite-infected hepatocytes presented a significantly higher level of apoptosis than those infected with RAS, a difference that may be explained by the observed longer survival and development time of RAS within the host cell (**Figure 3. B and D**) (van Dijk *et al*., 2005). It appears that, the longer the attenuated parasite develops and survives within the host cell, the longer it is protected from apoptosis, maybe because the parasite, by arresting at an early stage of EEF development, lacks the capability of expressing factors that are essential for host cell apoptosis prevention, or by not developing further, ends up degenerating and subsequently, stops expressing the factors responsible for the protection. Surprisingly, it seems that within an infectious dose of WT parasites, some fail to prevent host cell apoptosis, maybe because they are deficient in some stage of their development, and end up degenerating (Leirião *et al*., 2005b; van Dijk *et al*., 2005). Nevertheless, this appears to be normal, occurring to a low percentage of WT parasites within an infectious dose.

IMMUNE RESPONSES INDUCED BY RAS AND GAS

As previously mentioned, immunization with live attenuated parasites elicits robust and long-lasting immune responses that completely protect against further infections. However, the nature of these immune responses is still not fully understood, although it appears that these may precisely be generated as a result from the fact that both RAS and GAS parasites fail to prevent host cell apoptosis.

For practical and ethical reasons, the vast majority of the studies aiming to understand the immune responses triggered by RAS and GAS immunization protocols have been performed using *Plasmodium* parasite rodent model systems, mainly *P. berghei* and *P. yoelii* with laboratory mice from two major backgrounds, BALB/c and C57BL/6. Collectively, results indicate that the sterile immunity generated by RAS is of a multi-factorial nature, involving both humoral and cellular immune responses, respectively targeting the sporozoite and the infected hepatocyte (Jobe *et al*., 2009). Although it includes both $CD4^+$ and $CD8^+$ T cells responses, the protective immune response was reported to be primarily mediated by intrahepatic interferon-γ (IFN-γ) producing effector memory $CD8^+$ T cells (T_{EM}), accompanied by the presence of central memory $CD8^+$ T cells (T_{CM}) that endure homeostatic proliferation, forming a reservoir of memory T cells (Berezon *et al*., 2003). The importance of $CD8^+$ T cell response is supported by the fact that immune protection by RAS is major histocompatibility class 1 (MHC-I)-dependent (White *et al*., 1996).

It has been argued that the success of the protection conferred by RAS and GAS, is owed to the fact that they elicit immune responses that are broad-based against multiple antigen targets, both from the sporozoite and early EEF developmental stages (Hoffman and Doolan, 2000). Nevertheless, evidence that the antibody responses induced were predominantly directed against the antigenic

repetitive central domain of the CSP (Zavala *et al*., 1986), as well as that adoptive transfer of CSP-specific $CD8^+$ or $CD4^+$ T cell clones was able to confer full protection against WT parasite sporozoite challenge (Romero *et al*., 1989; Rodrigues *et al*., 1991; Renia *et al*., 1993), led to the hypothesis that RAS-induced immune responses might be mediated by few immunodominant antigens, such as the CSP. In fact, this has prompted the development of subunit vaccines essentially based on CSP, such as the RTS,S and Sf66 vaccines (see above). However, the modest efficacies obtained by these vaccines, along with incongruous results obtained recently in two independent reports (Kumar *et al*., 2006; Grüner *et al*., 2007), question the importance of immune responses against CSP in the induction and acquisition of sterile protection.

Using BALB/c mice tolerant to *P. yoelii* CSP, therefore devoid of T cell dependent responses against CSP, Kumar *et al*., 2006 reported that CSP is a crucial antigen for the efficacy of RAS immunization. However, Grüner *et al*., 2007 demonstrated that sterile protection against *P. berghei* malaria can be induced independently of specific T cell immune responses to CSP. They have shown that BALB/c mice could be fully immunized by *P. berghei* RAS whose endogenous CSP had been replaced by the *P. falciparum* CSP, which does not elicit minimal T cells responses in those mice. Moreover, Mauduit *et al*., 2009 corroborated these later results, and actually proposed that this disparity could be due to the fact that immune responses against CSP and non-CSP antigens are induced differently in the two rodent malaria species. They have suggested that in *P. berghei* immune responses against non-CSP antigens, responsible for the sterile protection, are rapidly induced after RAS immunization, while in *P. yoelii* the responses arising rapidly are against CSP, being the ones targeting non-CSP antigens only achieved after several boosts with RAS. Nevertheless, it is still assumed that CSP is indeed the immunodominant antigen regarding the humoral responses, being the anti-CSP antibodies, generated after *P. yoelii* RAS, *uis3*$^-$ or *uis4*$^-$ parasite immunizations, able to neutralize infective sporozoites (Kumar *et al*, 2009).

As mentioned, RAS-induced protective immunity is mainly mediated by IFN-γ producing $CD8^+$ T_{EM} cells. $CD8^+$ T cells eliminate target cells mostly by engaging the perforin/granzyme B pathway to induce targeted cell apoptosis (cytotolytic activity). Here, through a pore formed by perforin, granzyme B is delivered to the targeted cell cytoplasm where it activates pro-apoptotic factors that ultimately lead to caspase-3 activation, or directly activates caspase-3 (James and Green, 2004). Surprisingly, protection against infection was successfully achieved in perforin as well as in granzyme B deficient mice after RAS immunization, suggesting that $CD8^+$ T cells were primarily killing infected hepatocytes through other mechanism (reviewed in Vaughan *et al*., 2010). This scenario is actually logical if we take in consideration that infected hepatocyte apoptosis is inhibited by the developing parasite, and that this is one possible reason for the development of IFN-γ mediated $CD8^+$ T cell immune response against infected cells, since IFN-γ affects the parasite indirectly, by inducing nitric oxide (NO) production in the infected cell. Nevertheless, cytolytic elimination of infected hepatocytes has also been reported, but appears to be less predominant than the IFN-γ mediated $CD8^+$ T cell response (van de Sand *et al*., 2005).

HOW ARE RAS AND GAS IMMUNE RESPONSES TRIGGERED? – DCS COME INTO PLAY

When infected cells undergo apoptosis, vesicles loaded with parasite antigens are formed (apoptotic bodies), that can thereafter be phagocytosed by antigenic presenting cells (APCs) such as macrophages and dendritic cells (DCs), and be further presented to the immune system, activating the subsequent specific immune responses. It has been hypothesized that this is precisely how protective immunity conferred by RAS or GAS is prompted (Leirião *et al*., 2005b; Jobe *et al*., 2009).

DCs are key players in the coordination of immune responses against a variety of pathogens, and are separated into several cellular subsets, each with unique functional activities. For instance, the cCD8α$^+$ DC subset is efficient in cross-presenting exogenous antigens (from extracellular sources, like apoptotic bodies) associated with MHC-I molecules to CD8$^+$ T cells, activating them (Jobe *et al*., 2009). Several pieces of evidence have suggested that DCs play an important role in the establishment of protective immunity induced by RAS, as mice devoid of DCs failed to develop protective immunity (Jung *et al*., 2002), and adoptive transfer of DCs loaded with *P. yoelii* CSP into naïve mice resulted in the generation of partial protective immunity (Bruña-Romero and Rodriguez, 2001). Moreover, two elegant studies have revealed strong evidences that DCs are actually the vehicle that connects the apoptotic infected hepatocytes and the protective immunity, mainly mediated by CD8$^+$ T cells responses (Leirião *et al*., 2005b; Jobe *et al*., 2009).

Leirião *et al*., 2005b showed, by means of immunofluorescence observations of liver histological sections from BALB/c mice, that DCs are recruited to the liver early after infection with *P. yoelii* sporozoites or RAS. This fast recruitment of DCs to the site of infection may be due to the local inflammatory response that is induced by the wounded and dying cells during sporozoite migration, and that may actually be suitable for DCs further maturation, since proper stimulatory signals may be present. Furthermore, this study also found macrophages and DCs carrying vesicles with parasite antigens at 6h p.i., when RAS-infected hepatocyte apoptosis was firstly reported. These vesicles were confirmed to be from apoptotic infected cell origin and not from single sporozoites since they contained Hsp70, a parasite protein not expressed in the sporozoite but in the EEF stages (Kumar *et al*., 1993), mouse albumin, a protein present within the hepatocyte cytoplasm, and activated caspase-3. Altogether, these results strongly suggest that indeed, apoptotic bodies originated from apoptotic RAS infected hepatocytes origin are phagocytosed by DCs in the liver. However, the study did not address whether these DCs were able to activate the effective CD8$^+$ T cell responses, a subject that was further analyzed by Jobe *et al*., 2009.

In Jobe *et al*., 2009, C57BL/6 mice were immunized with a *P. berghei* RAS immunization protocol, comprising a prime immunization and two subsequent boosts, during which the levels of DCs and CD8$^+$ T cells were followed, both in the liver and in the spleen. They showed that the cCD8α$^+$ DC population in the liver, nearly absent in naïve mice, accumulated during the course of the repeated immunizations with RAS, reaching the maximum level after the second boost, and that in fact, this coincided with the expansion of CD8$^+$ T_{EM} cells in the liver. The levels of both cells also increased in the spleen, but this was not as marked as in the liver. Moreover, they showed that this cCD8α$^+$ DC subset was indeed the primary inducers of the CD8$^+$ T_{EM} cell response through an experiment in which the co-culture of cCD8α$^+$ DCs from immunized mice, with CD8$^+$ T cells from naïve mice, lead to the acquisition of the IFN-γ secreting T_{EM} phenotype by the CD8$^+$ T cells. The cCD8α$^+$ DC subset was also

shown to exhibit high levels of MHC-I and to be an efficient producer of IL-12, the key cytokine promoting the activation of $CD8^+$ T cells. In fact, activation of $CD8^+$ T cells by liver $cCD8\alpha^+$ DCs from immunized mice was dependent on both MHC-I and IL-12. To finally proof that these liver $cCD8\alpha^+$ DCs were indeed responsible for the activation of the $CD8^+$ T_{EM} cell responses, they were adoptively transferred to naïve mice and actually, were able to confer protective immunity against further challenges with small doses of *P. berghei* sporozoites. Facing these results, the authors propose that liver $cCD8\alpha^+$ DCs cross-present *P. berghei* pre-erythrocytic antigens derived from apoptotic *P. berghei* RAS infected hepatocytes to liver $CD8^+$ T cells, activating them into IFN-γ secreting $CD8^+$ T_{EM} cells. Nevertheless, they were not able to completely infer whether this priming occurs in the liver, in the draining lymph node or in the spleen.

Recently, it was shown that *P. yoelii* CSP persists for more than 8 weeks after immunization with RAS, not because of parasite persistence as an arrested pre-erythrocytic stage, but because the antigen is transferred to other cell types, such macrophages and DCs present in the liver, spleen and lymph nodes (Cockburn *et al*., 2010). This antigen persistence appears to be able to enhance immune responses to the parasite by maximizing the size of the $CD8^+$ T_{EM} cell population, inducing proliferation of $CD8^+$ T_{CM} cell population, and by recruiting and promoting differentiation of naïve $CD8^+$ T cells from the thymus, into $CD8^+$ T_{EM} cells (Cockburn *et al*., 2010).

Antigen persistence may therefore be one of the factors accounting for the observed long-lasting durability of the protective immunity induced by attenuated sporozoites, and an additional evidence for the importance of DCs in the establishment and persistence of such immune response.

AIM

The aim of the present project was to test the hypothesis that apoptosis of infected hepatocytes with attenuated parasites, either RAS or GAS, is essential for the establishment of complete protective immunity, known to be induced by immunization with these parasites, against further challenges with infectious sporozoites.

To address this question, C57BL/6 background mice deficient in caspase-3 (Casp3KO mice), which, as already mentioned, is a key player in numerous apoptotic pathways, were immunized with either *P. berghei* RAS or *P. berghei p36p*$^-$ sporozoites. Challenge infections were further performed with either green fluorescent protein (GFP) – expressing *P. berghei* ANKA (*Pb*GFP, Franke-Fayard *et al*., 2004) or luciferase – expressing *P. berghei* ANKA (*Pb*Luci, Ploemen *et al*., 2009) sporozoites. In these parasites, the GFP and Luciferase genes are under the control of strong housekeeping gene promoters, being constitutively expressed in a growth responsive manner throughout the respective parasites life cycle. This feature allows the establishment of a correlation between the parasite load in the liver with the expression of GFP or luciferase in the liver of infected mice when assesses through appropriate established methodologies, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) (Bruña-Romero *et al*., 2001), and real-time *in vivo* imaging (Ploemen *et al*., 2009), respectively. The level of infection in the liver was used as a means to infer acquisition of immunity, although the efficiency of the immune response was only ultimately assessed by following the appearance and progression of the blood stage infection, by counting the percentage of parasites in the mice peripheral blood (parasitemia) (Mueller *et al*., 2005a).

In order to exclude the involvement of a deficient immune system responses in the outcome of Casp3KO mice immunization, bone marrow chimeric mice, either expressing caspase-3 exclusively in haematopoietic cells (Casp3KO mice reconstituted with C57BL/6 mice bone marrow) or in non-hematopoietic cells (C57BL/6 mice reconstituted with Casp3KO mice bone marrow), were generated, and immunized with *Pb*GFP-RAS.

Moreover, a breakthrough methodology to detect apoptosis *in vivo*, that combines fluorescent probes with a pan-caspase inhibitor, was also used to compare the level of apoptosis in the liver of mice infected with RAS and NIrr sporozoites.

MATERIALS AND METHODS

MICE

Six to twelve-week old male and female C57Bl/6 mice (from Instituto Gulbenkian de Ciência, Charles River Laboratories, or Harlan Ibèrica) and C57BL/6 background caspase-3 deficient mice (Casp3KO mice) (Kuida *et al.,* 1996) (bred in the Animal House of the Instituto de Medicina Molecular – IMM) were used. In each experiment, groups were formed by mice from the same age range, gender and, in the case of the C57Bl/6 mice, from the same supplier, to exclude variation within groups due to either age, gender or supplier related heterogeneity. Casp3KO mice were genotyped by tail genomic DNA Polymerase Chain Reaction (PCR) using primers (5'-TGACTGGGCACAACAGAC-3'; 5'-TTTCTCGGCAGGAGCAAG-3') targeting a 271 bp fragment of the neomycin resistance cassette used to generate them (Kuida *et al.,* 1996). The following amplification programme was used: 95ºC/5 min of initial denaturation, followed by 25 cycles of 95ºC/30 sec, 61ºC/30 sec and 72ºC/17 sec, and a final round of 72ºC/10 min to allow complete elongation of the PCR product. Protocols involving live animals were approved by the Animal Care Committee of the IMM.

PARASITES

Green Fluorescent Protein (GFP) – expressing *Plasmodium berghei* ANKA (*Pb*GFP, parasite line 260 cL2) (Franke-Fayard *et al.,* 2004), genetically attenuated *P36p* deficient GFP – expressing *P. berghei* ANKA (*Pbp36p*$^{-}$, parasite line 274 cL1.1) (van Dijk *et al*., 2005), and Luciferase – expressing *P. berghei* ANKA (*Pb*Luci, parasite line 676 m1cL1) (Ploemen *et al.,* 2009) sporozoites were used. Sporozoites were obtained by removal and disruption of salivary glands from freshly dissected 21-35 days – infected female *Anopheles stephensi* mosquitoes, bred at the Insectary of the IMM. After dissection, salivary glands were collected in Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Invitrogen), further disrupted, and passed through a 70 µm pore diameter cell strainer. Sporozoite's concentration was then determined by counting their number in a Neubauer chamber (Average of sporozoites per quadrant x 10^4 x dilution factor), that was previously maintained in a moist chamber for 10 minutes. Radiation attenuated *Pb*GFP sporozoites (*Pb*GFP-RAS) were obtained by exposing freshly obtained *Pb*GFP sporozoites to a 16 krad dose of γ-radiation (Orjih *et al*., 1982) on a Compagnie Oris Industrie IBL 437C irradiator (in all experiments involving mice) or MDS Gamma Cell 3000 *Elan* irradiator.

*Pb*GFP-RAS FITNESS – *IN VITRO* GLIDING, MIGRATION, INVASION AND DEVELOPMENT ASSESSMENT

For gliding motility assessment, glass coverslips were coated with 10 µl/ml of anti-circumsporozoite protein (CSP) 3D11 antibody (Ab) and incubated for 1h at 37ºC. Coverslips were then washed with PBS and 30.000 sporozoites were added per coverslip in complete RPMI 1640 medium (Gibco/Invitrogen). Subsequently, they were centrifuged for 5 min at 3000 rpm, and incubated for 30 min at 37ºC. Coverslips were then washed with PBS, fixed for 15 min in 4% paraformaldehyde (PFA) at room temperature (RT), and further incubated for 30 min with 10 % fetal calf serum (FCS, Gibco/Invitrogen). Afterwards, they were incubated for 1h with 0,03 mg/ml anti-CS 3D11 Ab, followed by 1h incubation with a goat anti mouse (GAM) alexa fluor 488 Ab (Invitrogen), both at RT. Finally, the

coverslips were washed with PBS, and mounted on slides with Fluoromount (Southern Biotech). Gliding motility was assessed by counting the average number of circle trails left by single sporozoites per coverslip (15 fields, 200x amplification) on a Leica DM5000B Widefield Fluorescence Microscope.

For migration, invasion and development appraisement, flow cytometry (FC) was used, as described in Prudêncio *et al*., 2008. Huh7 cells, a human hepatoma cell line, were cultured and maintained in complete RPMI medium (Gibco/Invitrogen) as described. For each assay, an average of 70.000 cells/well was seeded on 24-well plates the day before infection. Cells were infected with an average of 30.000 sporozoites/well, further centrifuged for 5 min at 3000 rpm, and incubated at 37ºC and 5% of CO_2 for 2h (migration and invasion), or 48h (development). In the case of migration evaluation, 2 mg/ml Dextran tetramethylrhodamine 10 000 MW, lysine fixable (fluoro-ruby) (Molecular Probes/Invitrogen), in RPMI complete medium, were added to the Huh7 cells immediately prior to infection. At the appropriate time point post infection (p.i.), cells were washed in PBS, incubated with trypsin (Gibco/Invitrogen) for 5 min at 37ºC, and collected in 10% FCS. Cells were then centrifuged for 5 min at 1200 rpm, further re-suspended in 2% FCS, and analyzed on a Becton Dickinson (BD) FACScalibur Flow Cytometer with the appropriate settings for GFP and Dextran tetramethylrhodamine detection. Data acquisition and analysis were performed as described in Prudêncio *et al*, 2008, using CELLQuest Pro (Becton Dickinson) and FlowJo (version 6.4.7, Tree Star, Inc 1997-2006) software packages, respectively.

P. berghei INFECTION PROGRESSION IN CASP3KO MICE

Groups of C57BL/6 and Casp3KO mice were infected intravenously (i.v.) in the retro-orbital venous plexus with 10.000 *Pb*GFP or *Pb*Luci sporozoites, depending on whether Quantitative Real-Time Reverse Transcription PCR (qRT-PCR), or *in vivo* Real Time Imaging, respectively, were employed to measure liver stage infection. In experiments where infection was allowed to proceed to the blood stage, Giemsa-stained thin peripheral blood smears were prepared from individual mice from day 3 up to 3-4 weeks after infection. The percentage of parasitized erythrocytes (parasitemia) was determined microscopically (Number of parasitized erythrocytes / Number of total erythrocytes x 100).

IMMUNIZATION WITH *Pb*GFP-RAS OR *Pb*P36P$^-$GAS AND PARASITE CHALLENGE EXPERIMENTS

Groups of C57BL/6 and Casp3KO mice were immunized with either *Pb*GFP-RAS or *Pbp36p*$^-$ sporozoites, as previously described (Orjih *et al*., 1982, Douradinha *et al*., 2007). Both groups were injected i.v. with three immunization doses, 8 days apart, of 10.000 *Pb*GFP-RAS or 2.000 *Pbp36p*$^-$. Eight to 12 days after the last immunization, mice were challenged i.v. with 10.000 *Pb*GFP or *Pb*Luci sporozoites. In every immunization and challenge experiments, a group of non-immunized C57BL/6 mice was challenged, at the same time as the immunized groups, to confirm the infectivity of the challenge dose. In experiments where infection was allowed to proceed to the blood stage, blood parasitemia was followed as already described.

QUANTIFICATION OF *Pb*GFP LIVER INFECTION BY qRT-PCR

Livers were collected from mice 40-44h after infection/challenge with *Pb*GFP sporozoites, and mechanically homogenized in denaturating solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7; 0,5% N-Lauroyllsarcosine; and 0,7% of freshly added β-mercaptoethanol, in Diethylpyrocarbonate (DEPC)-treated water). Total RNA was further extracted using RNeasy Total

RNA Mini Kit (Qiagen), and converted into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's protocols and the amplification programme: 25ºC/10 min, 55ºC/30 min and 85ºC/5 min. Equal amounts of cDNA per sample were then amplified by qRT-PCR using Finnzymes F-410XL DyNAmo™ HS SYBR® Green qPCR Kit with primers targeting *P. berghei* 18S rRNA (5'-AAGCATTAAATAAAGCGAATACATCCTTAC-3'; 5'-GGAGATTGGTTTTGACGTTTATG TG-3'), or mouse hypoxanthine guanine phosphoribosyl transferase (HPRT) housekeeping gene (5'-GTAATGATCGTCAACGGGGGAC-3'; 5'-CCAGCAAGCTTGCAACCT TAACCA-3') transcripts. The amplification reaction was performed in an Applied Biosystems 7500 Fast Real-Time PCR System, using the instrument's standard ramp speed and the amplification programme: 50ºC/2 min and 95ºC/10 min, followed by 50 cycles of 95ºC/15 sec and 60ºC/1 min, and one cycle of 95ºC/15 sec, 60ºC/1 min, 95ºC/30 sec and 60ºC/15 sec. Parasite load was obtained by calculating the relative amounts of *P. berghei* 18S cDNA copies (although primers targeting GFP cDNA could also be used since the expression of both genes correlates positively, Prudêncio *et al*., 2008) against HPRT cDNA copies, per mouse liver.

The level of $CD8^+$ T cells in the liver was assayed using the same procedure with primers targeting CD8 cDNA copies (5'-GCTGGTAGTCTGCATCCTGCTTC-3'; 5'-TTGCTAGCAGGCTATCAGTGTTGT G-3'). $CD8^+$ T cells load was obtained by calculating the amounts of CD8 cDNA relative to HPRT cDNA, per mouse liver.

QUANTIFICATION OF *Pb*LUCI LIVER INFECTION BY REAL-TIME *IN VIVO* IMAGING

At 40-44h after infection/challenge with *Pb*Luci sporozoites, mice were examined by Real Time Imaging as described in Ploemen *et al.,* 2009. Briefly, mice were anesthetized with isoflurane (IsoFlo, Veterinária Esteves) and immediately injected subcutaneously (in the neck) with D-Luciferin dissolved in PBS (0,15 mg/g, Caliper Life Sciences). Precisely 5 min after, mice were intra-peritoneally (i.p.) injected with 200 µl of an anaesthesia mixture comprising Ketamine (1,2% of Imalgene 1000, Merial) and Xylazine (0,8% of 2% Rompun, Bayer), diluted in PBS, and placed inside the imaging camera of the *in vivo* Imaging System (IVIS® Lumina Imaging System). Bioluminescence imaging was acquired exactly 10 min after D-Luciferin injection, with a 12.5 cm field of view (FOV), binning factor of 4, and an exposure time of 180 seconds. Quantitative analysis of bioluminescence was performed by measuring the luminescence signal intensity using the region of interest (ROI) settings of the Living Image® Software (version 3.0). The ROIs were set to measure the abdominal area (that was previously shaved using chemical depilatory, Veet®) at the location of the liver. ROI measurements were expressed in total flux of photons, and were used to infer liver stage infection.

CASP3KO BONE MARROW CHIMERIC MICE

Chimeric mice were generated as described in Cunha-Rodrigues *et* al., 2007, with slight adaptations. In two separate sessions of 450 rad (2h apart, minimum), recipient C57BL/6 and Casp3KO mice were lethally γ-irradiated with an overall dose of 900 rad. Bone marrow from donor C57BL/6 and Casp3KO mice was removed from the major leg bones, femur and tibia, by flushing in 4% FCS at 4ºC, with a 27G syringe. The bone marrow was gently flushed until a homogenized suspension of cells was obtained. This was then passed through a 100 µm diameter pore cell strainer, and cell concentration was calculated by counting their number in a Neubauer chamber (Average of cells per quadrant x 10^4 x dilution factor). Cells were then centrifuged for 5 min at 1200 rpm, and re-

suspended in PBS in a volume suitable to i.v. inject 6,5 x 10^6 cells per recipient mouse. Recipient C57BL/6 mice were subsequently reconstituted with bone marrow cells from donor Casp3KO mice (ChimC57BL/6), while recipient Casp3KO mice were reconstituted with bone marrow cells from donor C57BL/6 (ChimCasp3KO). An additional group of C57BL/6 mice that was also lethally γ-irradiated but was not reconstituted, and was left depleted from bone marrow cells, to serve as irradiation control group. These three groups were treated with a wide spectrum antibiotic, Trimetoprim/Sulfametoxazol (40 mg + 200 mg, Bactrim® paediatric suspension, Roche) diluted in the drinking water (1:50), from day 2 before irradiation until 4 weeks after irradiation. Chimeric mice were allowed to recover for 8 weeks, to allow the irradiation control group to succumb naturally, before any procedure was performed on them. Afterwards, chimeric mice were immunized with *Pb*GFP-RAS, and subsequently infected with *Pb*Luci, as described above. Depletion of circulating cells and reconstitution with donor cells was confirmed in all chimeric mice by PCR of blood genomic DNA. Blood was collected from the tail and DNA extracted using DNeasy Blood and Tissue Kit (Qiagen). PCR was performed using two sets of primers that amplify sequences either from the knock out (same pair of primers and amplification programme used for genotyping, as described in *Mice*) or the wild type allele (5'-TGTCATCTCGCTCTGGTACG-3'; 5'-AAATGACCCCTTCATCACCA-3'; 1250 bp), the later with the amplification programme: 95ºC/5 min of initial denaturation, followed by 35 cycles of 95ºC/1 min, 58ºC/1 min, and 72ºC/1 min 30 sec, and a final round of 72ºC/10 min, to allow complete elongation of the PCR product.

IN VIVO DETECTION OF APOPTOSIS IN THE LIVER

Groups of C57BL/6 and Casp3KO mice were i.v. infected with either *Pb*GFP or *Pb*GFP-RAS (100.000 – 280.000 sporozoites/mouse). As negative controls, two groups of C57BL/6 mice were not infected, while as a positive control, another group of C57BL/6 mice was i.v. injected with 5 µg/20 g of purified mouse anti-Fas/CD95/APO-1 monoclonal antibody (α-Fas, Pharmingen) (Imao *et al*., 2005). At 5h 30 min p.i., the infected groups, the α-Fas injected group, and one of the non-infected groups were injected i.v. with the red fluorescent probe SR-FLIVO™ (SR-VD-FMK, sulforhodamine-B conjugated valylalanylaspartic acid fluoromethyl ketone; ImmunoChemistry Technologies, LLC), prepared according to the manufacturer's protocol. In each experimental set, mice were injected with similar amounts of SR-FLIVO™ (13,1 – 16,4 µg/mouse). SR-FLIVO™ was allowed to circulate for about 30 min, after which mice were i.p. injected with a Ketamine and Xylazine anaesthesia mixture, and placed inside the imaging camera of the *in vivo* Imaging System (IVIS® Lumina Imaging System). Fluorescence imaging was acquired using DsRed as excitation and ND3 as emission filters, with a FOV of 12,5, a binning factor of 8, and an exposure time of 1 sec. Subsequently, livers were removed, and analysed again by fluorescence imaging, using the same image settings as before.

TRANSGENIC PARASITE – PRO-CASPASE-2 EXPRESSING AND EXPORTING *P. berghei*

The pro-caspase-2 insert (1371 bp) was amplified by PCR from C57BL/6 mice liver cDNA, using designed primers with inserted restrictions sites (**Table 1.**), and the amplification programme: 95ºC/5 min of initial denaturation, followed by 35 cycles of 95ºC/30 sec, 56ºC/30 sec and 72ºC/1 min 30 sec, and a final round of 72ºC/10 min. Meanwhile, CSP promoter (pCS, 1047 bp) and pexel motif (242 bp) inserts were amplified from *P. berghei* ANKA genomic DNA, using primers designed with a site mutagenesis and inserted restrictions sites (**Table 1.**), and the respectively amplification programmes:

95ºC/5 min of initial denaturation, followed by 35 cycles of 95ºC/30 sec, 43ºC/30 sec and 62ºC/1 min, and a final round of 62ºC/10 min (pCS); 95ºC/5 min of initial denaturation, followed by 35 cycles of 95ºC/30 sec, 41ºC/30 sec and 62ºC/20 sec, and a final round of 62ºC/10 min (pexel motif).

Gene (accession number)	Primer	Sequence	Introduced Restriction Sites
Pro-Caspase-2 (NM_007610)	Forward	CTC**GGATCC**GAAATGGCGGCGCCGAGC	BamHI
	Reverse	TTT**TCTAGA**C**GCGGCCGC**ACGTGGGTGGGTAGCCTGG	NotI and XbaI
CS Promoter (PBANKA_040320)	Forward	AAA**GAATTC**AAATATGCAAGATGG	EcoRI
	Reverse	CAT**ATTTAAAT**ATATGCG	SwaI
CS PEXEL motifs (PBANKA_040320)	Forward	GCATAT**ATTTAAAT**ATGAAG	SwaI
	Reverse	TTT**GGATCC**TTCGGGAGCATCGGC	BamHI

Table 1. Primers used to amplify the target region of each gene. The restriction sites introduced to allow plasmid construction were highlighted in the sequences, and the restriction enzymes identified. A site mutagenesis was also inserted in both CS promoter and CS PEXEL motifs pair of primers (highlighted in red).

In total, six plasmids (Plasmid A to Plasmid F, **Figure 9.**) were planned to be constructed in a modular fashion. All constructs were assembled on two backbone plasmids, the pcDNA 3.1 (+) plasmid (Invitrogen), to allow *in vitro* tests, and the pVIII plasmid (courtesy of Gunnar Mair's laboratory, **Figure 4.**), that will be ultimately transfected into parasites. Briefly, to construct the six plasmids, both inserts and backbone plasmids were incubated with the appropriate restriction enzymes and compatible buffers (all from Fermentas, **Table 1.**) for 1h at 37ºC, and further purified using the High Pure PCR Product Purification Kit (Roche). Next, ligation was performed, by incubating both insert and plasmid with ligase and respective buffer (Fermentas), at proportions of 5:1 and 3:1, overnight at 15ºC. Afterwards, competent *E. Coli* DH5α cells (Invitrogen) were added to the ligation mix, incubated for 20 min at 4ºC, and later subjected to a heat chock 45 sec at 42ºC, followed by a short incubation of 2 min at 4ºC. Subsequently, cells were grown in liquid lysogeny broth (LB) medium without ampicillin for 45 min at 37ºC, and further plated on LB agar medium supplemented with ampicillin (1000x concentrated, 100 µl/ml) overnight. Single grown colonies were then collected and grown in liquid LB medium supplemented with ampicillin (1000x concentrated, 100µl/ml) overnight. Afterwards, cultures were centrifuged for 5 min at maximum speed, and the pellet resuspended in 260 µl of STET (8g of sucrose, 5g of TritonX-100, 10 ml of 0,5 M EDTA, and 5 ml of Tris-HCL 1 M pH 8), to which 40 µl of lysozyme was freshly added. Immediately after, it was boiled for 1 min, and centrifuged at maximum speed for 15 min at 4ºC. The pellet was then discarded, and 400 µl of isopropanol were added to the supernatant. The resulting mixture was thoroughly vortexed and further incubated for 10 min at 4ºC, for plasmid precipitation. The pellet was then dried and further resuspended in 100 µl of water

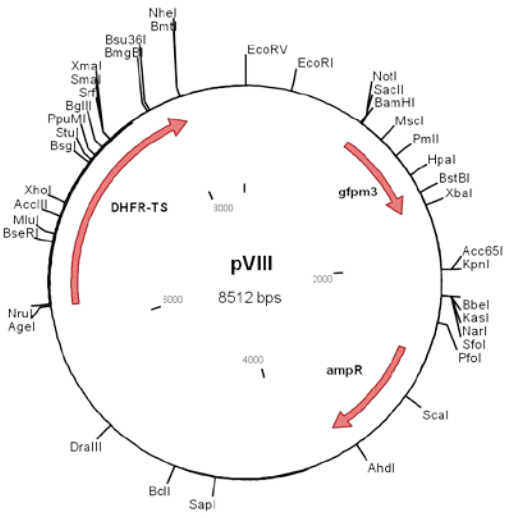


Figure 4. pVIII plasmid. Courtesy of Gunnar Mair's laboratory. Has a DHFR-TS and ampicillin resistance markers. Restriction sites are represented.

with 20 µl/ml of RNase. The correct ligation of the insert into the isolated plasmid was further confirmed both by enzymatic restriction and sequencing (Stabvida).

Pro-caspase-2 activity was tested *in vitro*, using Hepa 1-6 cells, a C57L/J mouse hepatoma cell line, that were maintained in DMEM medium supplemented with 10% FCS, 1% Penicillin/Streptomycin and 1% L-glutamine (all from Gibco/Invitrogen), at 37ºC and 5% of CO_2. For each experiment, non transfected cells, cells transfected with the constructed plasmid (Plasmids A, D or E), and cells transfected with pcDNA 3.1 (+) plasmid alone were used. An average of 80.000 cells per well of a 24-well plate were transfected with 0,4 µg of plasmid and 1,8 µl of Fugene 6® Transfection Reagent (Roche), according to the manufacturer protocol. Cells were then plated on glass coverslips in a 24-well plate and incubated at 37ºC and 5% of CO_2 until analysis was performed at 24, 25, 26, 30 and 50h later. Upon analysis, cells were incubated with DMEM medium with 1:20 of AlamarBlue® (Invitrogen) for 1h 30 min at 37ºC, after which were analysed on a Infinite 200 plate reader using the following parameters: excitation wavelength of 530 nm, emission wavelength of 590 nm, number of flashes of 10, gain of 100 and mode top reading.

After testing *in vitro* if the final construct is functional (Plasmids D and E), Plasmid C or F (depending on whether the construct functions when fused to GFP), will be linearized by cutting with SwaI and transfected into *P. berghei* ANKA blood schizont stages through electroporation, as described in Janse *et al*., 2006. Blood schizont stages will then be injected into BALB/c mice and further selected by treatment with pyrimethamine. Further, transgenic parasites will be genotyped using primers targeting the inserts of the construct (**Table 1.**). Afterwards, mosquitoes will be infected with the transgenic parasite, where its development will be characterized; sporozoites will be characterized for *in vitro* gliding motility, migration, invasion and development, as well as their ability to infect and develop in C57BL/6 mice. If the construct is efficiently expressed and induces apoptosis of the infected hepatocytes, C57BL/6 will be infected with the transgenic parasites (the number of infections will be determined) and followed-up for the establishment of protective immunity against further challenge with infectious sporozoites (*Pb*GFP or *Pb*Luci).

STATISTICAL ANALYSIS

Statistical analysis was performed using unpaired Student *t* Test, on Microsoft Excel.

RESULTS AND DISCUSSION

INFECTION WITH *P. berghei* ANKA – A VARIABLE OUTCOME FOR CASP3KO MICE

Thamnomys surdaster, an African tree rat, is the natural host of *Plasmodium berghei*. Because it is not amenable to routine use in the laboratory, several inbred strains of mice have been evaluated to best replace it for laboratory studies, such as the BALB/c, DBA and C57BL/6 strains. Scheller *et al*., 1994, demonstrated that the system C57BL/6-*P. berghei* ANKA is an optimal model to study the biology of pre-erythrocytic stages of the parasite (that take 40-44h to fully develop in this model), and the cellular protective mechanisms elicited after immunization with *P. berghei* radiation attenuated sporozoites (*Pb*RAS). Moreover, C57BL/6 mice are susceptible to the development of cerebral malaria (CM) by *P. berghei* ANKA erythrocytic stages, a very reproducible phenotype characterized by neurologic signs, such as paralysis, ataxia, convulsions and coma, accompanied by a relatively low percentage of infected erythrocytes (parasitemia), below 10%, at the time of death. Generally, CM occurs within 7-9 days p.i., with a cumulative mortality of nearly 90%. Nonetheless, about 10% of the mice do not develop CM and eventually succumb during the following 3 to 4 weeks from severe anaemia and hyperparasitemia, without neurological signs (Jennings *et* al., 1997; Lou *et al.*, 2001).

Therefore, in the present work, C57BL/6 mice and C57BL/6 background mice deficient in caspase-3 (Casp3KO mice) were used, along with several *P. berghei* ANKA parasite lines: expressing the GFP or luciferase genes (*Pb*GFP or *Pb*Luci, respectively), attenuated by irradiation (*Pb*GFP-RAS), and attenuated by genetic modification (*Pb*p36p^{-}).

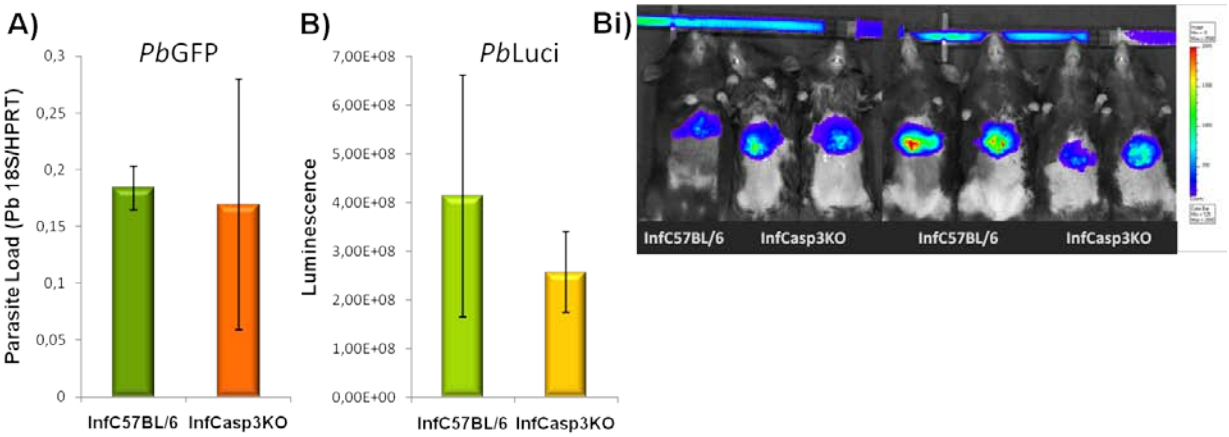
Prior to addressing if Casp3KO mice could be successfully immunized by attenuated parasites, the outcome of *P. berghei* ANKA infection of Casp3KO and C57BL/6 mice, regarding both liver and blood stages of infection, was compared. Pre-erythrocytic stages of *Plasmodium* are known to prevent host cell apoptosis both during their early and late development (Leirião *et al*., 2005a; van de Sand *et al*., 2005). Nevertheless, some parasites in an infective population are unable to prevent the host cell from undergoing apoptosis (Leirião *et al*., 2005b; van Dijk *et al*., 2005). Hence, it would be expected that in Casp3KO mice, which lack a crucial player of the major apoptotic pathways (Carmen and Sinai, 2007), the parasite load in the liver would be higher, or at least, similar to that observed in C57BL/6 mice, as even the parasites that may fail to prevent host cell apoptosis would be able to fully develop.

In two independent assays, both groups of mice were infected i.v. with 10.000 sporozoites, *Pb*GFP or *Pb*Luci, and liver stage infection was assessed at 40-44h p.i., by qRT-PCR (**Figure 5. A**) or real time *in vivo* imaging (**Figure 5. B**), respectively. Since the analysis of liver infections with *Pb*Luci by whole body imaging shows a good correlation with qRT-PCR analysis of extracted livers from infected mice (Ploemen *et al*., 2009), it is possible to compare the two assays. Unfortunately, it was not possible to draw any definite conclusions from both assays, as there were no statistically significant differences between the two experimental groups (**Figure 5. A and B**). Interestingly, the Casp3KO mice group showed an unexpectedly lower level of parasite load than the average level of the C57BL/6 group, and the reasons why remain unclear. One reason may be the existence of other caspase-3-independent apoptotic pathways, as well as of other highly related and even structurally similar to caspase-3 effector caspases, like caspase-7, that may compensate for caspase-3 loss and

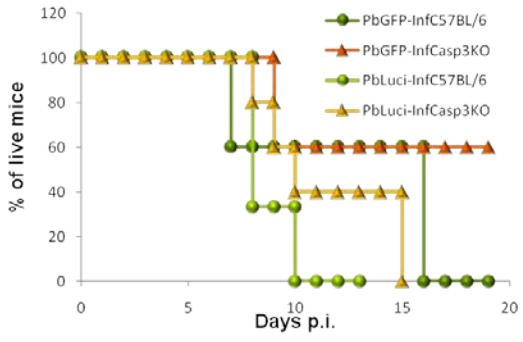
lead to apoptosis of caspase-3 deficient cells (Lakhani *et al*., 2006). This could be further evaluated by immunofluorescence detection of other effector caspases like, for example, activated caspase-7 in infected Casp3KO primary hepatocytes.

Unlike qRT-PCR, which requires sacrificing the mice in order to mesaure liver parasite load, *Pb*Luci real-time *in vivo* imaging enables assessing liver stage infection in live mice that can be further followed to monitor the development of blood stage infection (Ploemen *et al*., 2009). Therefore, blood stage infection was followed in an adicional group of mice infected with *Pb*GFP, and the original groups infected with *Pb*Luci (**Figure 5. C**). Blood stage infection was followed by counting individual mice parasitemia in thin peripheral blood smears stained with Giemsa, that strongly stains the DNA of the parasite and faintly the shape of the erythrocyte, which is an enucleated cell (Fleischer, 2004).

From the groups infected with *Pb*GFP, only two (n=5) C57BL/6 mice developed clear symptoms of CM and perished within the CM time window, with an average parasitemia of 3,36% (**Figure 5. C and D**). The other 3 mice did not develop neurological signs, and perished by day 16 p.i., most likely due to the development of anaemia. Interestingly, none of the infected Casp3KO mice (n=5) developed CM symptoms, although 2 mice did succumb within the expected time window with an average parasitemia of 4,37% (**Figure 5. C and D**). The other 3 mice did not develop any neurological signs and died only at day 20 p.i. with severe anaemia and hyperparasitemia, in the range of the 40% (**Figure 5. C and D**).



C)



D)

	Number	Day p.i.	Parasitemia (%)	Cause
*Pb*GFP-InfC57BL/6	2 (5)	7	3,36	CM
	3 (5)	16	14,36	Hyperparasitemia
*Pb*GFP-InfCasp3K0	2 (5)	9	4,37	Not from CM
	3 (5)	20	40,02	Hyperparasitemia
*Pb*Luci-InfC57BL/6	All (3)	7-9	4,07	CM
*Pb*Luci-InfCasp3K0	2 (4)	7-9	5,82	Not from CM
	2 (4)	15	21,48	Hyperparasitemia

Figure 5. Casp3KO mice infection with *P. berghei* ANKA. A) *Pb*GFP liver stage infection at 40h p.i., assessed by qRT-PCR. The two groups are not significantly different (p>0,05, Student *t* test). **B)** *Pb*Luci liver stage infection at 44h p.i., assessed by real time *in vivo* imaging. The two groups are not significantly different (p>0,05, Student *t* test). **Bi)** Rainbow images of luminescence in livers of live mice after luciferin injection, showing the relative levels of luminescence ranging from low (blue), to medium (green) and to high (yellow/red), and from where the graphic in B) was derived. **C)** Survival curve representing the % of live mice from the same experiments as A) and B) throughout the blood stage infection follow up. **D)** Table representing the day, average % of parasitemia and the cause of death of the mice represented both in B) and C).

In the groups infected with *Pb*Luci, all C57BL/6 mice developed CM within the expected time window with an average parasitemia of 4,07%, while the group of Casp3KO mice behaved similarly as in the above discussed assay (**Figure 5. C**). Interestingly, there was no observable correspondence between the levels of parasite load in the liver and the progression of blood stage infection, although it would to be expected that mice with higher parasite load in the liver would develop severe disease or higher parasitemia earlier than the others with a lower load.

Together, these results suggest that Casp3KO mice are resistant to CM development by *P. berghei* ANKA, although they eventually perish from one of two outcomes, either within the CM time window and parasitemia range, without neurological sings, or with severe anaemia and hyperparasitemia. The detection of activated caspase-3 in the brains of *P. berghei* ANKA infected mice with clinical signs of CM, mainly in neurons, oligodendrocytes, and endothelial cells, has suggested that apoptosis of endothelial cells may represent a critical step for the development of CM, and that the neurological signs and symptoms may be, at least in part, attributable to the apoptotic degeneration of neurons and glia in advanced stages of CM (Lackner *et al*., 2006). Since the Casp3KO mice lack caspase-3, and the active form of this enzyme appears to have an important role in the establishment and development of CM, it is plausible that this may be one of the possible reasons for the observed phenotype.

P. berghei ANKA RAS – THE EXPECTED PHENOTYPE

*Pb*RAS are very efficient in generating immunity in C57BL/6 mice (van Dijk *et al*., 2005). Nevertheless, the application of radiation to attenuate the parasite poses reservations regarding its safety and reproducibility, as improper irradiation of parasites may allow them to remain infectious and cause disease (Ballou, 2007). Properly attenuated *Pb*RAS retain their ability to invade hepatocytes comparable to the non-irradiated (NIrr) sporozoites, although they fail to fully develop within the host cell (Suhrbier *et* al., 1990). To ensure that the parasites herein used were being properly attenuated, and to test the reproducibility of two different irradiators, a simple *in vitro* assay comparing *Pb*GFP-NIrr and *Pb*GFP-RAS, either obtained by irradiation of *Pb*GFP sporozoites on a Compagnie Oris Industrie IBL 437C irradiator (*Pb*GFP-IrrHosp) or on a MDS Gamma Cell 3000 *Elan* irradiator (*Pb*GFP-IrrIMM), was performed.

During gliding, sporozoites continuously release vesicles covered with circumsporozoite protein (CSP) from their cell surface membrane (Frevert *et al*., 2005). When gliding on artificial surfaces, such as glass coverslips, sporozoites leave behind the CSP vesicles in the shape of a trail that corresponds to their patterns of movement (Stewart and Vanderberg, 1988). To access sporozoite gliding motility, glass coverslips were coated with an anti-CSP antibody (3D11), where sporozoites were allowed to glide freely for 30 min, and were further incubated with the same anti-CSP antibody. Gliding motility fitness was then inferred by counting the average number of circle trails left by single sporozoites (**Figure 6. A**). The results showed that the standard radiation dose (16 krad) used to attenuate sporozoites has an effect on the parasite's gliding motility, as both *Pb*GFP-IrrHosp and *Pb*GFP-IrrIMM sporozoites showed a significant reduction in gliding motility, of 57 and 46%, respectively, comparing to the *Pb*GFP-NIrr sporozoites (**Figure 6. A**).

Once in the liver parenchyma, sporozoites migrate through several hepatocytes, disrupting their cellular membranes (that can later be resealed, ensuring wounded hepatocytes survival), while

traversing through them. Afterwards, sporozoites eventually invade a final hepatocyte with the formation of a parasitophorous vacuole (PV), where they dedifferentiate into a developing exoerythrocytic form (EEF) (Mota *et al*., 2001). By infecting hepatoma cells in medium containing rhodamine dextran (RD), a dye that is rapidly incorporated by traversed cells and further trapped within after membrane reseal (Mota *et al*., 2001), with *Pb*GFP parasites that constitutively express GFP throughout their entire life cycle (Franke-Fayard *et al*., 2004), it was demonstrated that these three biological processes efficacy (migration, invasion and development) can be successfully inferred by flow cytometry (Prudêncio *et al*., 2008). Flow cytometry is a technique that allows a fast and quantitative examination of cellular characteristics of thousands of individual cells in a heterologous population, including the detection and differentiation cellular subpopulations (Mittag and Tárnok, 2009). Briefly, flow cytometry analysis of cellular populations is based on the resulting light scattered pattern of each individual cell and additionally, if labelled with a fluorophore, on the fluorescence intensity that is emitted from the labelled cell, representing the amount of fluorochrome molecules per each cell (Mittag and Tárnok, 2009).

At 2h p.i. (Mota *et al*., 2001), Huh7 cells, a hepatoma cell line, infected with *Pb*GFP-NIrr, *Pb*GFP-IrrHosp, or *Pb*GFP-IrrIMM, in RD containing medium, were analysed by flow cytometry, and the frequency of RD or GFP positive cells was determined (**Figure 6. B**). As expected, no significant differences were observed between the three groups, confirming that RAS keep their ability infect cells comparable to the normal parasites (**Figure 6. B**). At 48h p.i., a time point suitable to observe *P. berghei* EEF development in these hepatoma cells (Prudêncio *et al*., 2008), the frequency of GFP cells, an indication of whether infected cells are dying with time, and the intensity of GFP per cell, as a result of parasite development, were assessed (**Figure 6. C**). Regarding the number of infected cells, which in overall decreases with time as a result of the multiplication of cells in culture, there is a decrease in the frequency of GFP positive cells infected with RAS parasites comparing to the NIrr one, although this is only significant in the *Pb*GFP-IrrHosp infected cells. This may be explained by the fact

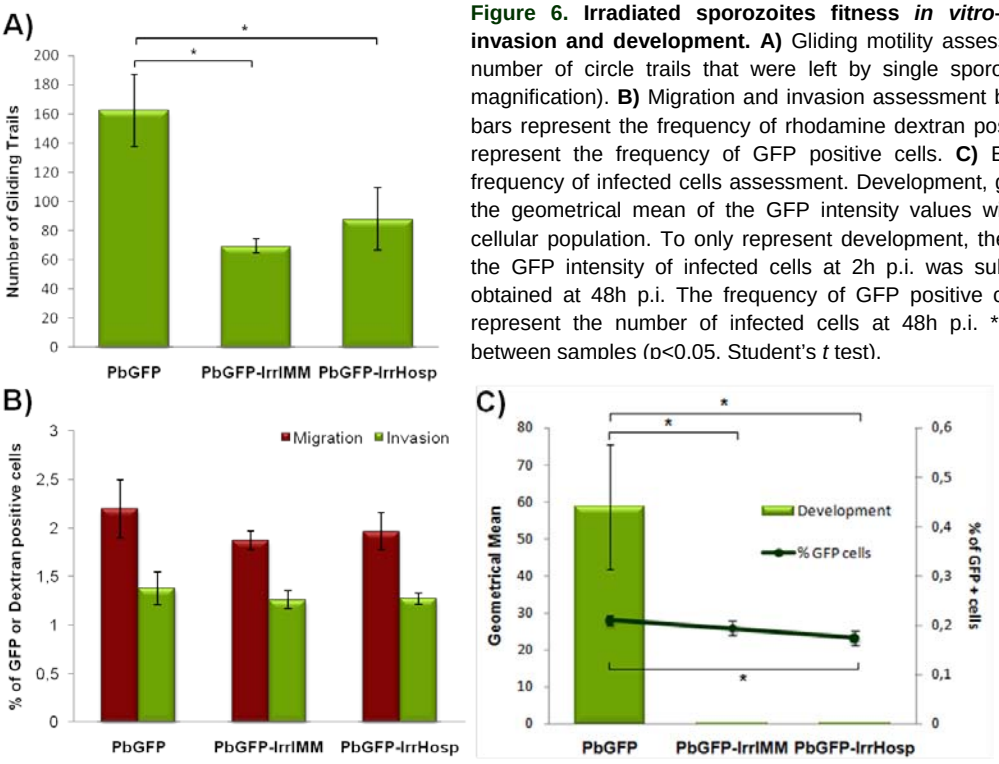


Figure 6. Irradiated sporozoites fitness *in vitro*– gliding, migration, invasion and development. A) Gliding motility assessment by counting the number of circle trails that were left by single sporozoites (15 fields, 20x magnification). **B)** Migration and invasion assessment by flow cytometry. Red bars represent the frequency of rhodamine dextran positive cells; green bars represent the frequency of GFP positive cells. **C)** EEF development and frequency of infected cells assessment. Development, green bars, is given by the geometrical mean of the GFP intensity values within the GFP positive cellular population. To only represent development, the geometrical mean of the GFP intensity of infected cells at 2h p.i. was subtracted to the values obtained at 48h p.i. The frequency of GFP positive cells, dark green dots, represent the number of infected cells at 48h p.i. * Significant difference between samples ($p<0.05$, Student's *t* test).

that from 6h p.i. onwards, RAS-infected hepatocytes start succumbing to apoptosis due to the incapacity of the parasite to prevent it (Leirião *et al*., 2005b; van Dijk *et al*., 2005). As EEFs develop, more copies of GFP are present per cell, and therefore, a higher GFP intensity is detected per infected cell. The fluorescence intensity spectrum of a heterogeneous population is plotted as a fluorescence intensity histogram, from which the extent of EEF development can be inferred by determining the geometrical mean of the distribution. Confirming what was previously reported (Suhrbier *et* al., 1990; Prudêncio *et al*., 2008), both *Pb*GFP-IrrHosp and *Pb*GFP-IrrIMM showed a severe development arrest, presenting GFP intensity values similar to the ones obtained at 2h p.i. (**Figure 6. C**), meaning that the invasive parasites did not developed, while the *Pb*GFP-NIrr developed as expected.

Altogether, the results confirm that RAS maintain their capacity to migrate and invade hepatocytes as the NIrr sporozoites, although their gliding motility was significantly affected. As expected, both radiators were able to completely attenuate the parasites, as development was completely arrested in both groups of RAS, demonstrating that the irradiation process is reproducible using the two distinct irradiator machines.

IMMUNIZATION WITH ATTENUATED SPOROZOITES – RAS AND GAS

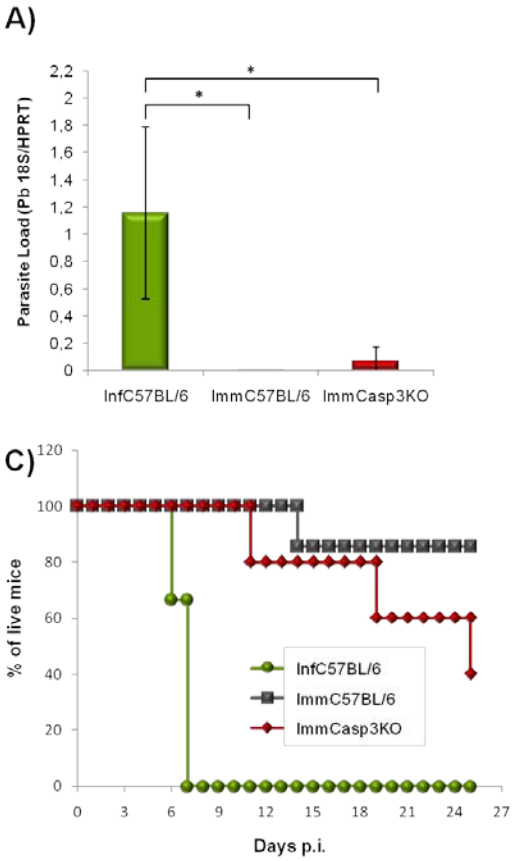
In two independent experiments, one group of C57BL/6 (ImmC57BL/6) and another of Casp3KO (ImmCasp3KO) mice were immunized according to an established three-dose immunization protocol, comprising a prime immunization followed by two boosts of 10.000 *Pb*GFP-RAS or 2.000 *Pbp36p*$^-$ sporozoites, and subsequently challenged with 10.000 sporozoites (Douradinha *et al*., 2007). Upon challenge, an additional group of non-immunized C57BL/6 mice was also infected, as a control group for infection (InfC57BL/6).

In the experiment where mice were immunized with *Pb*GFP-RAS, the challenge was performed with *Pb*GFP sporozoites and, at 40h p.i., liver parasite load was assessed by qRT-PCR. Similar to what was previously reported (Douradinha *et al*., 2007), a marked reduction in liver stage development of *Pb*GFP parasites (>99%) was observed in ImmC57BL/6 mice when compared to the non-immunized, InfC57BL/6 mice, which presented a normal level of infection (**Figure 7. A**). Such significant decrease of parasite development may indicate that sterile protection against infectious challenge was achieved in ImmC57BL/6 mice. Interestingly, a significant reduction in parasite load, around 94%, was also observed in the ImmCasp3KO mice group (**Figure 7. A**), although this was not as marked as the one observed in the ImmC57BL/6 group, possibly suggesting that immunity generated in the ImmCasp3KO mice might not be as efficient as the one generated in the ImmC57BL/6 group. Nevertheless, the difference observed between the immunized groups was not statistically significant, and sterile protection of ImmCasp3KO mice against the infectious challenge was not observed at this stage of parasite infection.

Moreover, CD8 copies in mice livers, extrapolated to represent the $CD8^+$ T cell load, were also quantified by qRT-PCR (**Figure 7. B**) to infer whether the protection conferred in Casp3KO mice was mediated by this T cell population. As expected, InfC57BL/6 mice shown a basal level of CD8 copies, representing the level of $CD8^+$ T cells normally present in the liver, as it was too soon for any adaptive immune response to be prompted, even one that could be possibly triggered by a normal infection. Furthermore, ImmC57BL/6 exhibited a significant increased level of CD8 copies, of approximately 3,3 times the one of InfC57BL/6 mice (**Figure 7. B**), corresponding to the cellular immune response that is

characteristically generated against challenge after immunization with *Pb*GFP-RAS. Moreover, this increase is in accordance with the drastic reduction of parasite load observed in these livers (**Figure 7. A**). Nevertheless, the same was not observed for the ImmCasp3KO group. Despite the reduced parasite load, these mice did not present an increased level of CD8 copies. Instead, their level was similar to the one observed in InfC57BL/6 mice, and significantly lower (nearly 2,7 times) than the one of the ImmC57BL/6 mice, suggesting that the reduction in parasite load observed in ImmCasp3KO mice is not mainly due to a $CD8^+$ T cell immune response. Nonetheless, it cannot be assumed that these $CD8^+$ T cells are targeting the parasitized cells. For confirmation, an IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay, using peptides from parasite antigens, like CSP, could be performed as previously described (Mauduit *et al*., 2009). Such an assay would allow testing cross-reactive T cell responses induced by the immunization regimen, through the quantification of parasite antigen specific IFN-γ producer $CD8^+$ T cells isolated from the spleen of the different mouse groups and, therefore, confirm if the increase of $CD8^+$ T cells in ImmC57BL/6 is composed by $CD8^+$ T cells specifically targeting the parasite. Besides, it would also be interesting to perform an Enzyme-linked immunosorbent assay (ELISA) to assess the levels of antibodies targeting parasite antigens, like CSP, induced by the immunization regimen (Mauduit *et al*., 2009). Essentially, an ELISA assay would allow us to assess if a humoral immune response is also involved and especially, if the reduction in liver infection in ImmCasp3KO mice is mediated by an antibody response.

Protective immunity was ultimately assessed by allowing the challenge infection to progress to the



D)

	Number	Day p.i.	Parasitemia (%)	Cause
InfC57BL/6	All (3)	6-8	5,18	CM
ImmC57BL/6	1 (7)	14	3,22	7 day delay, CM?
	6 (7)	-	-	-
ImmCasp3KO	1 (5)	11	5,08	Not from CM
	2 (5)	21-25	10-63,7	Hyperparasitemia
	2 (5)	-	0,15-0,30	Cleared

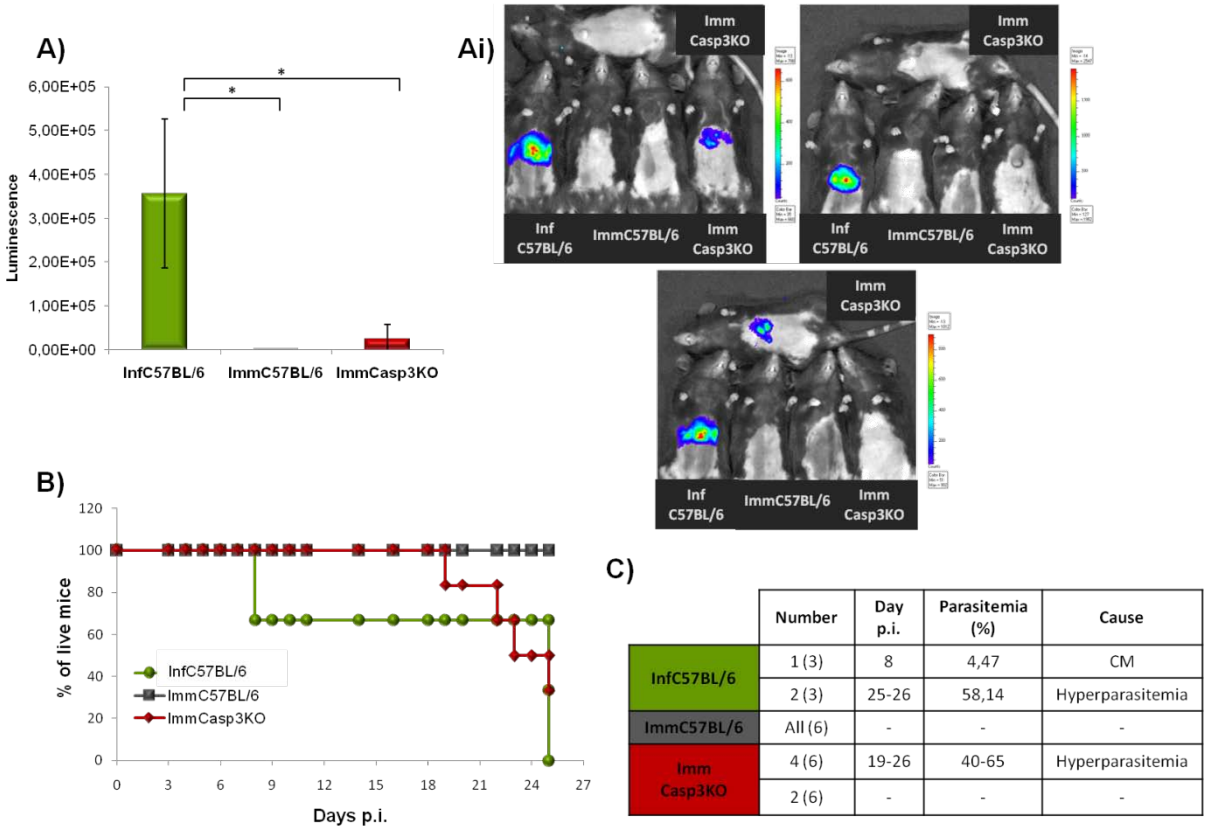
Figure 7. Immunization of Casp3KO mice with *Pb*RAS. A) Challenged *Pb*GFP liver stage infection at 40h p.i., assessed by qRT-PCR. **B)** Quantification of CD8 copies in infected livers, reflecting the amount of $CD8^+$ T cells, assessed at 40h p.i. by qRT-PCR. **C)** Survival curve representing the % of live mice throughout the blood stage infection follow up. **D)** Table representing the day, average % of parasitemia and the cause of death of the mice represented both in C). * Significant difference between samples ($p<0,05$, Student's *t* test).

blood stage as detection of developed blood stages of the parasite on blood smears from day 3 p.i. onwards constitute the most stringent readout for sterile protection against malaria infection (Mueller *et al*., 2005a). As expected, all InfC57BL/6 mice succumbed with CM within the expected time window (**Figure 7. C and D**). However not all ImmC57BL/6 mice were completely protected against infectious challenge. One (n=7) ImmC57BL/6 mouse was not properly immunized and developed malaria, nevertheless, with a prolonged pre-patent period compared to the InfC57BL/6 group (7 versus 3 days). Although the reasons behind this miss-immunization are unclear, one can speculate that it might have been due to an improper administration of the *Pb*GFP-RAS inoculae, rather than due to an improper irradiation of the sporozoites because, if it was the case, all mice would have developed disease during one of the immunization sessions. Nevertheless, this constitutes a good example of why there are so many reservations about using RAS as an antimalarial vaccine. Most importantly, none of the ImmCasp3KO mice were completely protected against infection (**Figure 7. C and D**), as all mice presented parasites in the blood by day 4 p.i., one day of delay comparing to the InfC57BL/6 mice. Interestingly, three phenotypes were observed. Similarly to what was observed in InfCasp3KO mice (**Figure 5. D**), one mouse (n=5) died within the CM time window without any neurological symptoms, while two others died several days later with severe anaemia and hyperparasitemia (**Figure 7. D**). Furthermore, two mice that had presented parasites in the blood (parasitemias of 0,15 to 0,30%) were able to clear the parasitemia and survive until the end of the experiment without reappearance of infection (**Figure 7. D**). The reasons behind this phenotype remain unknown, since immunization with attenuated sporozoites is recognized to be stage specific and to not confer immunization against blood stage forms of the parasite (van Dijk *et al*., 2005), and therefore, elimination of parasite's blood stages by specific RAS generated immunity in ImmCasp3KO mice is unlikely.

In the experiment where mice were immunized with 2.000 *Pbp36p*$^{-}$ sporozoites, challenge was performed with *Pb*Luci sporozoites and, 40h p.i., liver stage infection was assessed by real-time *in vivo* imaging. As expected, and similarly to what was observed for immunization with *Pb*GFP-RAS, ImmC57BL/6 mice showed a significant reduction (>99%) in liver stage development of *Pb*Luci parasites comparing to the InfC57BL/6 mice, while ImmCasp3KO mice presented a less marked decrease (<93%) (**Figure 8. A**). This suggests that immunity generated in ImmCasp3KO mice is less efficient than the one generated in ImmC57BL/6 mice. Indeed, when analyzing the data per mouse, only 2 of 6 mice presented a clear detectable liver stage infection (**Figure 8. Ai**), suggesting that some ImmCasp3KO mice may have been successfully immunized against further infections. Nevertheless, because parasite infection in the liver of these mice is *per se* variable, one can hypothesize that in mice where a normal infection would be low, the reduction of infection due to an even partially effective immune response could be enough to decrease parasite load to a level below the detection threshold of the real time *in vivo* imaging system. Therefore, protection was ultimately assessed by following the progression of infection in the blood. Both InfC57BL/6 and ImmC57BL/6 groups behaved as expected: all InfC57BL/6 mice presented parasites in the blood, and every ImmC57BL/6 mice were completely protected from infection (**Figure 8. B and C**). Nevertheless, only one (n=3) InfC57BL/6 mouse died from CM while the others succumbed from hyperparasitemia (50 to 70%) and severe anaemia (**Figure 8. B and C**). Unexpectedly, 2 (n=6) ImmCasp3KO mice were completely protected from infection, as they have never presented parasitemia until the end of the experiment and in fact,

had no detectable parasites in the liver (**Figure 8. A**, left and bottom image, top mice, and **C**). All other ImmCasp3KO mice succumbed from hyperparasitemia (around 60%) and severe anaemia after day 18 p.i. *Pbp36p*$^{-}$ sporozoites have been described to have a more severe deficiency than *Pb*RAS in preventing host cell apoptosis (van Dijk *et al.*, 2005), and in fact, this may account for their ability to confer complete immunity with such low dose of sporozoites. Possibly, this may allow *Pbp36p*$^{-}$ infected caspase-3 deficient hepatocytes to undergo apoptosis to a larger extent than when infected with *Pb*GFP-RAS, and therefore establish, although only in one third, complete immune protection against further challenges in ImmCasp3KO mice.

Casp3KO mice immunized with either *Pb*GFP-RAS or *Pbp36p*$^{-}$ show a marked decrease in liver stage infection. This is an interesting finding since it hypothesized that immunity generated by these parasites, primarily mediated by CD8^{+} T cells, is triggered by apoptotic infected cells (Leirião *et al*., 2005b; Jobe *et al*., 2009). Although caspase-3 deficiency can be compensated either by alternative independent pathways or by other effector caspases, caspase-3 has shown to be essential in certain processes associated with cell dismantling and further formation of apoptotic bodies (Porter and Jänicke, 1999). It is possible that in Casp3KO mice, although infected hepatocytes may undergo apoptosis independently from caspase-3, apoptotic bodies filled with parasite antigens from infected hepatocytes origin may not being formed, or at least, not at the same rate as in wild type mice. This could explain why ImmCasp3KO mice with *Pb*GFP-RAS did not developed a, at least detectable, CD8^{+} T cell mediated immune response, given that the uptake of parasite antigens filled apoptotic



	Number	Day p.i.	Parasitemia (%)	Cause
InfC57BL/6	1 (3)	8	4,47	CM
	2 (3)	25-26	58,14	Hyperparasitemia
ImmC57BL/6	All (6)	-	-	-
Imm Casp3KO	4 (6)	19-26	40-65	Hyperparasitemia
	2 (6)	-	-	-

Figure 8. Immunization of Casp3KO mice with *Pb*RAS. A) Challenged *Pb*Luci liver stage infection at 44h p.i., assessed by real time *in vivo* imaging. **Ai)** Rainbow images of luminescence in livers of live mice after luciferin injection, showing the relative levels of luminescence ranging from low (blue), to medium (green) and to high (yellow/red), and from where the graphic in A) was derived. **B)** Survival curve representing the % of live mice, the same from Ai), throughout the blood stage infection follow up. **C)** Table representing the day, average % of parasitemia and the cause of death of the mice represented both in C). * Significant difference between samples ($p<0,05$, Student's *t* test).

bodies by DC's has been associated with the generation of such response (Jobe *et al*., 2009). Nevertheless, immunization of Casp3KO mice confers them partial immunity against further infection, although the nature of the involved immune responses is still unknown, and requires further investigation.

IMMUNIZATION OF CHIMERIC MICE WITH *Pb*RAS

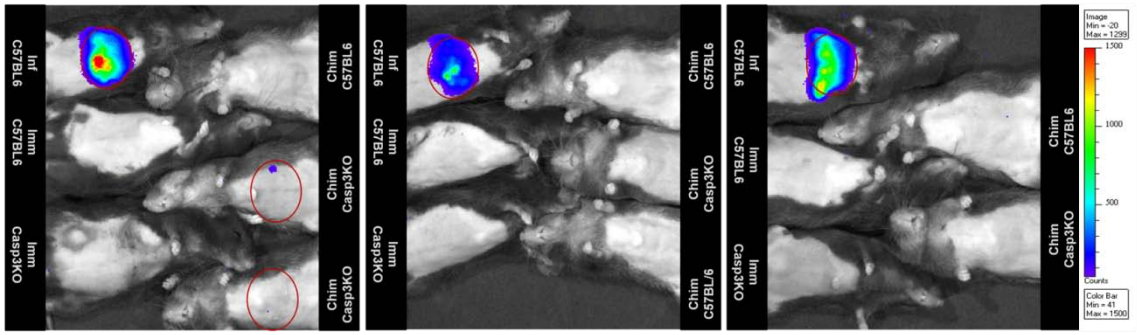
Since Casp3KO mice are deficient in caspase-3 in all their cells and not just in hepatocytes, one may argue that ImmCasp3KO mice are only partially protected because their immune system may be, to some extent, deficient, and not because of their lower apoptosis of infected liver cells. Nevertheless, the phenotype is unlikely to be due to a deficiency in the immune system, since both immature B and T cells of Casp3KO mice have been reported to undergo normal apoptosis during differentiation (reviewed in Porter and Jänicke, 1999). Moreover, Lakhani *et al*., 2006, have shown that both caspase-3 and caspase-7 are not necessary for the positive and negative selection of T cells, suggesting that alternative pathways might be involved. Together, these observations indicate that Casp3KO mice may have normal B and T cell mediated responses and that the partial immunity generated in this mice upon immunization with attenuated sporozoites may be, in fact, a result of their deficiency in the formation of apoptotic bodies from infected hepatocytes. Nonetheless, to exclude the involvement of a deficient immune system in the observed phenotypes in ImmCasp3KO mice, bone marrow chimeric mice, either expressing caspase-3 exclusively in haematopoietic cells (Casp3KO mice reconstituted with C57BL/6 mice bone marrow – ChimCasp3KO) or in non-hematopoietic cells (C57BL/6 mice reconstituted with Casp3KO mice bone marrow – ChimC57BL/6), were generated (Cunha-Rodrigues *et al*., 2007). The chimeric groups, along with a C57BL/6 (ImmC57BL/6) and another Casp3KO (ImmCasp3KO) groups were immunized with three doses of 10.000 *Pb*GFP-RAS, and further challenged with 10.000 *Pb*Luci sporozoites. A non-immunized C57BL/6 group was also infected. It was expected that both chimeric groups would respond to infection as their immunized control groups have responded in the previous experiments: Chim57BL/6 and ImmC57BL/6 mice would be completely immunized and no parasites in the liver and blood would be detected, while ChimCasp3KO and ImmCasp3KO mice would only be partially protected, and parasites would be detected in the livers and blood of these mice.

At 48h p.i., the InfC57BL/6 group presented a normal liver parasite load, whereas none of the immunized groups presented a clear detectable pre-erythrocytic infection signal (**Figure 9. A**), although two ChimCasp3KO mice showed a faint signal in the abdominal region where the liver localizes (**Figure 9. A**, left image). However, it is not accurate to assume that this fainted signal was in fact the parasite developing, as it is too low to have complete certainness. These results might suggest that all the immunized groups were successfully protected against subsequent infection, although some signal may be below the detection threshold of the real time *in vivo* imaging system and therefore, conclusions can only be taken by following the infection in the blood of these mice.

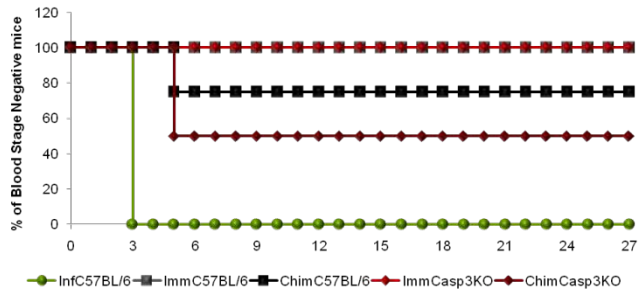
As expected, the InfC57BL/6 group presented parasitemia by day 3 p.i. (**Figure 9. B**), and developed CM symptoms by day 7. However, only one (n=3) died from CM. The two others recovered from the symptoms and perished later on by hyperparasitemia (**Figure 9. C**). Moreover, all ImmC57BL/6 mice were completely protected against infection, as well as 3 (n=4) of the ChimC57BL/6 mice. Unexpectedly, one ChimC57BL/6 mouse presented parasites in the blood,

although with a 2 day-delay in the pre-patency period comparing to InfC57BL/6 mice (5 versus 3 days) (**Figure 9. B**). Conversely to what was previously observed in ImmCasp3KO mice, all mice were protected against infection, as none presented any parasitemia until day 21 p.i., as well as two (n=4) of the ChimCasp3KO mice. Two other ChimCasp3KO mice presented parasites in the blood, also with a 2 day-delay pre-patent period, as was observed for the ChimC57BL/6 (**Figure 9. B**). This may indicate that even with a normal immune system, these mice are only partially protected because apoptosis of RAS-infected hepatocytes occurs in a lesser extent than in C57BL/6 cells. However, overall, these results do not allow us to take any definite conclusions, especially because the ImmCasp3KO mice, here as an immunization control, were completely protected from infection. Furthermore, the appearance of parasites in the blood of one ChimC57BL/6 mouse does not allow us to conclude whereas this was due to a deficiency in the Casp3KO origin immune system or because this mouse was improperly immunized.

A)



B)



C)

	Number	Day p.i.	Parasitemia (%)	Cause
InfC57BL/6	1 (3)	8	5,37	CM
	2 (3)	20	47,7	Hyperparasitemia
ImmC57BL/6	All (3)	-	-	-
ChimC57BL/6	3 (4)	-	-	-
	1 (4)	20	32,3	Hyperparasitemia
ImmC57BL/6	All (3)	-	-	-
ChimCasp3KO	2 (4)	-	-	-
	1 (4)	10	8,2	Not from CM
	1 (4)	23	38,4	Hyperparasitemia

Figure 9. Immunization of chimeric mice with *Pb*RAS. A) Rainbow images of luminescence in livers of live mice after luciferin injection, showing the relative levels of luminescence ranging from low (blue), to medium (green) and to high (yellow/red). Red circles (regions of interest) surrounding the abdominal region where the luminescence signal is. **B)** Blood stage negative mice curve, representing the day when parasitized mice had parasites in the blood. **C)** Table representing the day, average % of parasitemia and the cause of death of the mice represented in A).

Because the process of creating a chimeric mice is very long (8 weeks), and the immunization protocol takes 3 weeks to be completed, the mice herein used were older (5-6 months at infection) than the ones used in the previously described immunization experiments (2-3 months at infection). This might have had an influence on the observed outcomes, as the immune responses might differ as age progresses. An immunization experiment, with more mice per group and with the age range of the chimeric mice should therefore be performed in order to check whether the protection outcome is similar to the one observed in younger mice.

DETECTION OF APOPTOSIS *IN VIVO* – MICE INFECTED WITH *Pb*RAS HAVE HIGHER LEVELS OF APOPTOSIS IN THE LIVER

Recently, through the conjugation of a cell-permeant pan-caspase inhibitor, Z-VAD-fmk (carbobenzoxy – valyl-alanyl-aspartic acid – fluoromethyl ketone), that binds to the catalytic site of active caspases, with fluorescent probes, such as sulforhodamine (SR) and carboxyfluorescein (FAM), a non-toxic poly-caspase reagent for detection of apoptosis *in vivo* (Fluorescence in vIVO, FLIVO^{TM}), was developed (Griffin *et al*., 2007). Following i.v. injection in mice, FLIVO^{TM} circulates within the bloodstream, rapidly diffusing through cells. If active caspases are present, FLIVO^{TM} irreversibly establishes covalent bonds with them, inhibiting their enzymatic activity and thus progression of the apoptotic process, while unbound reagent is taken with the bloodstream and cleared within one hour after injection. As long as the cellular membrane is intact, FLIVO^{TM} remains inside the cell, causing the apoptotic cells to fluoresce, giving a direct measure of the caspase activity that was occurring when the reagent entered the cell (Immunohistochemistry, 2009). According to the manufacturer, FLIVO^{TM} fluorescence can be measured non-invasively by real-time *in vivo* imaging, or invasively, both by microscopic observation of thin tissue sections and flow cytometry (Immunohistochemistry, 2009).

Using SR-FLIVO^{TM}, we sought to detect differences in apoptosis in the livers of mice infected with *Pb*GFP or *Pb*GFP-RAS at 6h p.i., the time point when apoptosis of *Pb*RAS-infected hepatocytes was previously reported to occur (van Dijk *et al*., 2005). Therefore, mice were i.v. infected with 160.000 to 280.000 sporozoites of *Pb*GFP or *Pb*GFP-RAS, and 5h30min later were i.v. injected with SR-FLIVO^{TM}, which was allowed to circulate for 30 min, as recommended by the manufacturer (Immunohistochemistry, 2009).

In a first assay, three groups of C57BL/6 mice, non-infected (N.I.C57BL/6), infected with *Pb*GFP (InfC57BL/6), and infected with *Pb*GFP-RAS (Inf-IrrC57BL/6), and another group of Casp3KO mice infected with *Pb*GFP-RAS (Inf-IrrCasp3KO), were injected with SR-FLIVO^{TM} (**Figure 10. A**). Results from real-time *in vivo* imaging shown an increased level of apoptosis in Inf-IrrC57BL/6 mice comparing to all the other groups, although this was only statistically significant from the level observed in the N.I.C57BL/6 group (**Figure 10. A and Ai**). Nevertheless, these results suggest that *Pb*GFP-RAS parasites are unable to prevent host cell apoptosis. However, the level of apoptosis in Inf-IrrCasp3KO mice appears to be slightly higher than the one observed in the InfC57BL/6 mice, suggesting that in these mice some *Pb*GFP-RAS parasites are unable to prevent host cell apoptosis, and that other caspase-3 independent apoptotic pathways might be involved (**Figure 9, A**). Because an extra group of mice, both non-infected and non-injected with SR-FLIVO^{TM}, was not included as a negative control, it was not possible to exclude whether some of the signal observed, for instance in the nose and paws of mice, was due to the blood's auto-fluorescence, or to SR-FLIVO^{TM} still in circulation (**Figure 10. Ai**). Unfortunately, it was not possible to quantify the apoptotic cells either by microscopy and flow cytometry.

In another assay, all groups were constituted by C57BL/6 mice. Negative and positive controls were included, respectively, a group that was both not infected nor injected with SR-FLIVO^{TM}, and a group that was injected, 5h30min before SR-FLIVO^{TM}, with 5µg of α-Fas antibody, reported to cause massive liver failure within hours after injection as a result of the induction of hepatocyte apoptosis (Imao *et al*., 2006). The other three groups of mice were all injected with SR-FLIVO^{TM}, one non-

infected (N.I.C57BL/6), other infected with *Pb*GFP (InfC57BL/6) and other with *Pb*GFP-RAS (Inf-IrrC57BL/6) (**Figure 10. B** and **C**). Real time *in vivo* imaging analysis of the negative control mice revealed that blood auto-fluorescence signal is captured within the same emission signal range as SR-FLIVO^{TM} (**Figure 10. B**), demonstrating that the blood contributes with a background signal that must be in the future deducted from the experimental group's images. In order to avoid blood's auto-fluorescence, that could be masking SR-FLIVO^{TM} signal, livers were removed, washed in PBS, and further imaged again in the real time *in vivo* imaging system. Surprisingly, the simple wash with PBS was able to clear the background signal originated from the blood, and the differences between all the experimental groups was evident (**Figure 10. C and Ci**). Unfortunately, the positive control group did not behave as expected: mice injected with α-Fas antibody presented a lower level of apoptosis than all the other experimental groups, except for the InfC57BL/6 one (**Figure 10. C** and **Ci**). The reasons behind this incapability of the α-Fas antibody to induce massive apoptosis in the liver are unknown, although this might have been a consequence of the antibody decrease efficiency due to several freeze-thaw cycles, since the same antibody batch has been previously used in the same context with the expected outcome. Nevertheless, and although it is not statistically significant, Inf-IrrC57BL/6 mice appear to have a higher level of apoptosis comparing to all the other experimental groups, (**Figure 10.**

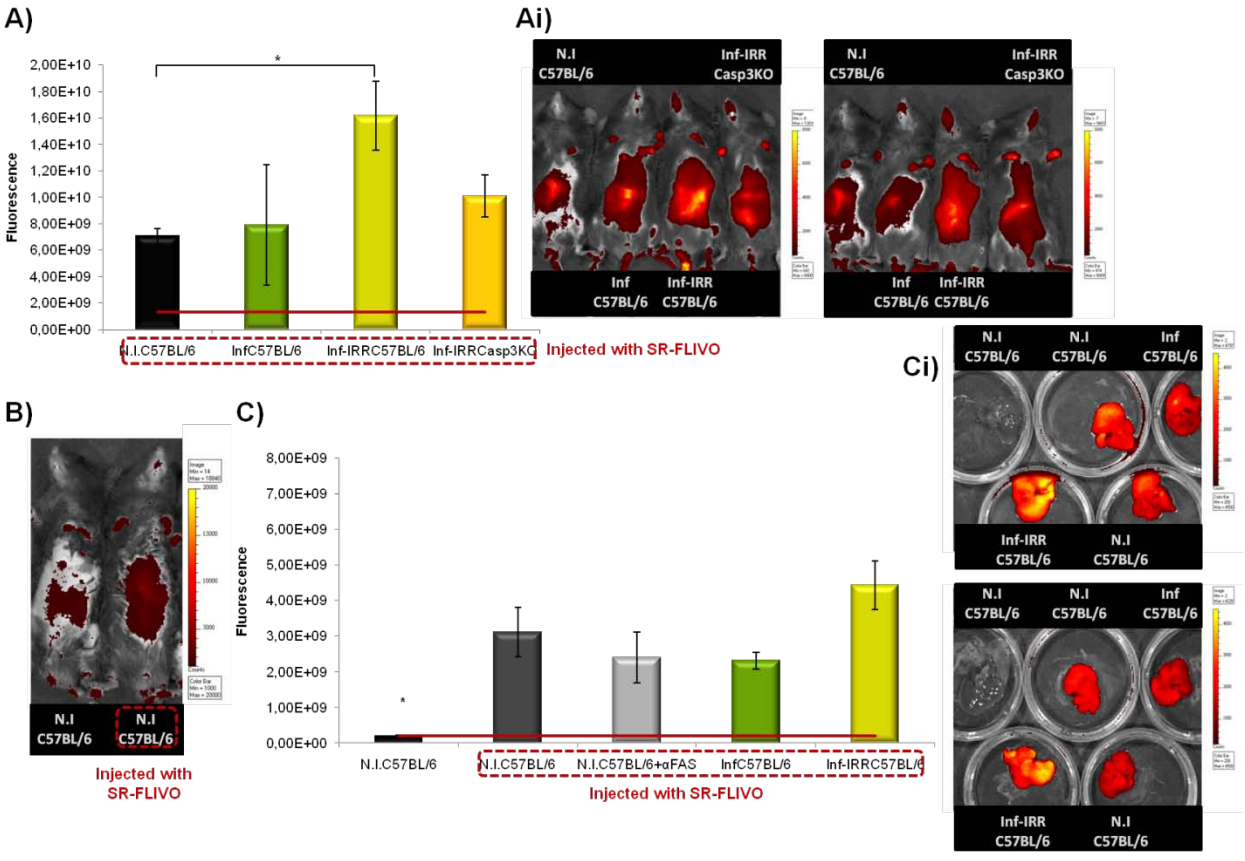


Figure 10. Detection of apoptosis *in vivo*. A Graphic representing the amount of fluorescence, corresponding to the amount of apoptosis, emitted from the abdominal region comprising the liver. Groups that were injected with SR-FLIVO^{TM} are within the red dotted box. Red line – background that was deduced from the red signal in the mice noses. **Ai** Red Hot images of fluorescence in livers of live mice 30 min after SR-FLIVO^{TM} injection, showing the relative levels of fluorescence ranging from low (dark red), to medium (bright red) and to high (orange/yellow), and from where the graphic in A) was derived. **B)** Red Hot images of blood's auto-fluorescence non-infected and non- SR-FLIVO^{TM} injected mouse (left), and non-infected but SR-FLIVO^{TM} -injected mouse. **C)** Graphic representing the amount of fluorescence emitted by extracted livers. **Ci)** Red Hot images of fluorescence in extracted livers 45 min after SR-FLIVO^{TM} injection, showing the relative levels of fluorescence ranging from low (dark red), to medium (bright red) and to high (orange/yellow), and from where the graphic in C) was derived. * Significant difference between samples ($p<0,05$, Student's *t* test).

C), supporting what was previously observed in the first assay (**Figure 10. A**). Interestingly, the InfC57BL/6 group seemed to show a lower, although not significant, level of apoptosis than the N.I.C57BL/6 mice injected with SR-FLIVO^{TM}. This might suggest that infection with infectious parasites inhibit host cell apoptosis, as previously reported (Leirião *et al*., 2005a, van de Sand *et al*., 2005) (**Figure 10. C**), although it is not known that with this amount of parasite dose the system is sensitive enough to detect such phenomenon. Due to technical problems, it was not possible to quantify the apoptotic cells either by microscopy and flow cytometry.

Together, these results appear to, in some extent, support previous observations (van Dijk *et al*., 2005; Leirião *et al*., 2005a; van de Sand *et al*., 2005): that RAS are unable to prevent the host cell to undergo apoptosis, and that infectious parasites prevent host cell apoptosis.

TRANSGENIC PARASITE – A *P. berghei* ANKA PARASITE EXPRESSING AND EXPORTING TO THE HOST CYTOPLASM A PRO-APOPTOTIC FACTOR

The hypothesis that the immunity generated by attenuated parasites relies on the formation of apoptotic bodies from infected hepatocytes (Leirião *et al*., 2005b; Jobe *et al*., 2009), together with the results from the immunizations of Casp3KO mice suggesting that caspase-3 mediated apoptosis is, at least in part, involved in the acquisition of such protective immunity, has prompt us to design a transgenic *P. berghei* parasite that would promote host cell apoptosis during liver stage infection. We aim to infect mice with this parasite and check whether protective immunity against further challenges, similar to the one established with the attenuated parasites, is generated. In order to successfully construct such parasite, three requirements were needed: a highly efficient pro-apoptotic factor, a strong liver stage specific promoter to express it, and an efficient mechanism to export it to the host cell cytoplasm.

Caspases are produced as pro-caspases, and require processing into two subunits to generate the fully active enzymes (Colussi *et al*., 1998). Caspases can be divided into two classes, depending on the length of their amino-terminal pro-domains which determine whether they are capable of auto-activation (class I), or require activation by other activated class I caspases (class II) (Colussi *et al*., 1998). Ideally, pro-caspase-3 would have been chosen, as a pro-caspase-3 expressing parasite would possibly invert the observed phenotype in the ImmCasp3KO mice and perhaps, confer complete immune protection in these mice. However, pro-caspase-3 is a class II caspase, and has been shown to be a poor inducer of cell apoptosis when transfected in mammalian cells (Colussi *et al*., 1998). Conversely, pro-caspase-2, a class I caspase, has been reported to successfully auto-activate and induce apoptosis in transfected mammalian cells (Colussi *et al*., 1998), and was therefore chosen as a suitable pro-apoptotic factor to construct the transgenic parasite.

Plasmodium spp. circumsporozoite protein (CSP) is restrictedly expressed in the parasite's pre-erythrocytic stages, coating the sporozoite's surface as well as the plasma membrane of the developing EEF. Moreover, CSP has been also found in the cytoplasm (peak at 1-3h post invasion) of infected hepatocytes, where it interacts with the host cell for its own benefit (Singh *et al*., 2007). Recently it was reported that CSP export to the hepatocyte cytoplasm, which requires the traversal of the parasitophorous vacuole membrane (PVM), is mediated by pexel motifs (*Plasmodium* export element), present in the protein's N-terminal region (Singh *et al*., 2007). Interestingly, these motifs are conserved among *Plasmodium* species CSP, although some like *P. berghei* and *P. falciparum*

possess two functional pexel motifs, while others, like *P. vivax* and *P. ovale*, only have one, allowing possibly the translation of findings in rodent models to human parasites.

In view of pro-caspase-2's efficiency in inducing apoptosis, the expression of CSP through a strong pre-erythrocytic stage specific promoter and its efficient export motif, we sought to design a *P. berghei* transgenic parasite expressing pro-caspase-2 fused with CSP pexel motif under the regulation of the CSP promoter (pCS). Once expressed, pro-caspase-2 would be exported to the hepatocyte cytoplasm where it would subsequently auto-activate and induce host cell apoptosis, resulting in the formation of apoptotic bodies loaded with parasite antigens.

In order to create a final plasmid, harbouring the fused pCS, pexel motif and pro-caspase-2 fragments construct, to be inserted into the parasite's genome (*P. berghei* ANKA strain), several intermediate plasmids were assembled (**Figure 11.** Plasmid A–F). All plasmids were designed to be constructed in a modular fashion to enable the replacement, insertion, deletion or exchange of fragments between plasmids. Therefore, the three inserts (pCS, pexel, and pro-caspase-2) were amplified with primers containing specific restriction sites present in the two used backbone plasmids (pcDNA 3.1 (+) and pVIII), to allow their further insertion in each plasmid. The construction of intermediate plasmids is fundamental, as it allows testing *in vitro* the functionality of the different parts of the construct, and whether the different inserts are efficiently expressed and functional when fused to each other. Overall, seven plasmids were planned. For the *in vitro* tests, inserts were introduced immediately downstream to the pcDNA 3.1 (+) plasmid T7 promoter, to construct Plasmids A, D and E. Plasmid A was aimed to test pro-caspase-2 ability to successfully induce apoptosis, while Plasmids D and E were constructed to test the functionality of pro-caspase-2 when fused with the pexel motif, and further with GFP, respectively (**Figure 11.**). In order to obtain the final plasmid to be transfected into the parasite, the pVIII plasmid, which contains one of the most commonly used selectable markers in *Plasmodium* transfection, the altered *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene, that confers resistance to the antimalarial drug pyrimethamine

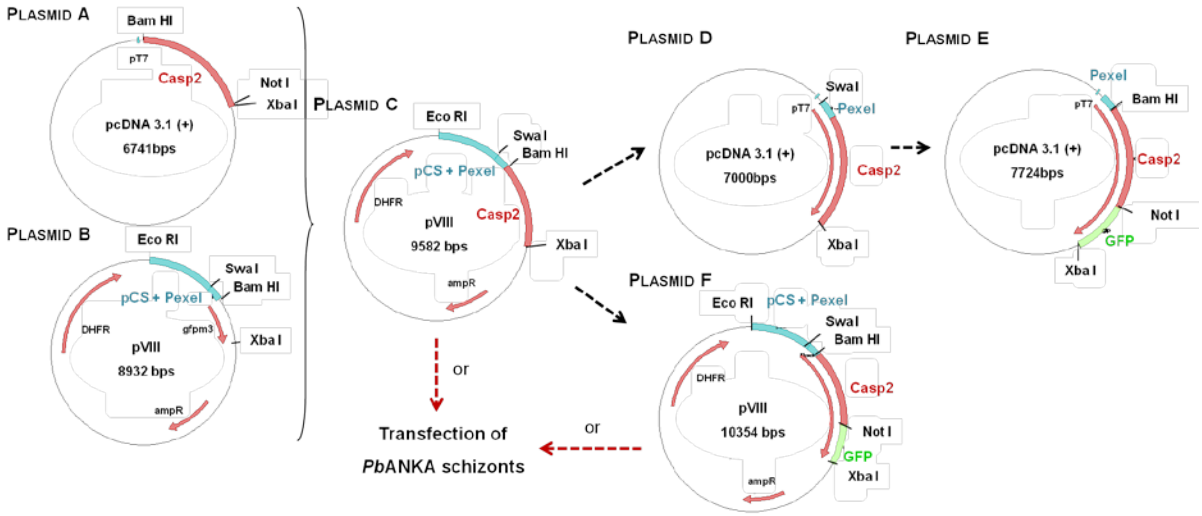


Figure 11. Transgenic, pro-apoptotic parasite. Plasmid A comprises the plasmid pcDNA 3.1 (+) with the inserted pro-caspase-2 gene. **Plasmid B** encompasses the plasmid pVIII with the inserted pCS and pexel motif. **Plasmid C** results from the insertion of the pro-caspase-2 fragment that was excised from Plasmid A, into Plasmid B to have has a final product the fused pCS, pexel motif and pro-caspase-2 construct. **Plasmid D** results from the insertion of the fragment pexel and pro-caspase-2 from Plasmid C into the pcDNA 3.1 (+) plasmid. **Plasmid E** outcomes from the insertion of a GFP fragment into Plasmid D, downstream to the pro-caspase-2 fragment. **Plasmid F** results from the insertion of the GFP fragment into Plasmid C, downstream to pro-caspase-2 fragment. Depending on whether the fused fragment of pCS, pexel and pro-caspase-2 with GFP still succeed to promote cell apoptosis, Plasmid C or Plasmid F will be transfected into *P. berghei* ANKA blood schizonts.

(Balu and Adams, 2007), was used. This allows the selection of the transgenic parasites in a heterogeneous parasite population with pyrimethamine, as the final construct inserted into the pVIII plasmid will be transfected in a pyrimethamine susceptible parasite line. Plasmid B was therefore constructed by inserting the fused pCS and pexel motif fragment into the pVIII plasmid (**Figure 11.**). If Plasmid A shows to be able to induce apoptosis in transfected cells, the pro-caspase-2 fragment is going to be excised and inserted into Plasmid B, downstream to the pexel motif fragment, resulting in a fused construct of the three fragments (Plasmid C). Because it possesses all the inserts fused and an inserted single cutter site within the pCS fragment, that allows its linearization and further transfection into the parasite, Plasmid C is ready to be transfected. Nevertheless, depending on whether Plasmid E testing reveals that pro-caspase-2 fused to GFP is functional, the GFP fragment will be further inserted into Plasmid C to generate Plasmid F, and therefore, instead of Plasmid C, it will be transfected into the parasite (**Figure 11.**). The tagging of the final construct, pCS, pexel motif and pro-caspase-2, with GFP would allow the visualization of caspase-2::GFP in the hepatocytes infected with the transgenic parasite. Upon transfection, by electroporation of blood schizonts, Plasmid C or F will be integrated into the parasite genome by homologous recombination, where the wild type CSP gene will be kept intact and functional, and the plasmid will be placed upstream to it. Afterwards, transgenic blood schizonts will be injected into BALB/c mice, which do not develop CM but instead, hyperparasitemia, to generate more transgenic parasites by selecting them with pyrimethamine. Subsequently, mosquitoes will be infected, the transgenic parasite life cycle characterized, and C57BL/6 as well as Casp3KO mice will be infected, with a number of doses to be defined, to infer if this transgenic parasite is able to confer them complete and sterile protection against further challenges with WT sporozoites.

Up to present, Plasmids A and B were successfully constructed. To test Plasmid A, Hepa 1-6 cells were transfected, and apoptosis was further assessed by AlamarBlue® assay at 24, 25, 26, 30 and 50h post transfection. The AlamarBlue® assay allows a very simple assessment of cell viability. Its active ingredient, resazurin, is a non-toxic cell permeable compound that, upon entering cells, is reduced to resorufin, a highly fluorescent compound. Fluorescence is then measured and its intensity correlates with the amount of living cells (Invitrogen, 2008). Unfortunately, no decrease in cell viability was reported in this assay, as at all time points cells transfected with Plasmid A had a similar level of fluorescence as the non-transfected cells and cells transfected with pcDNA 3.1 (+) alone, respectively, the negative and the transfection control groups (**Figure 12.**). Therefore, further microscopy tests, such as the TUNEL assay, and staining of active caspase-2 or caspase-3, or detection of pro-caspase-2 and active caspase-2 by western blot, must be performed to confirm whether pro-caspase-2 is being over expressed and activated, and if it is able to induce apoptosis of the transfected cells.

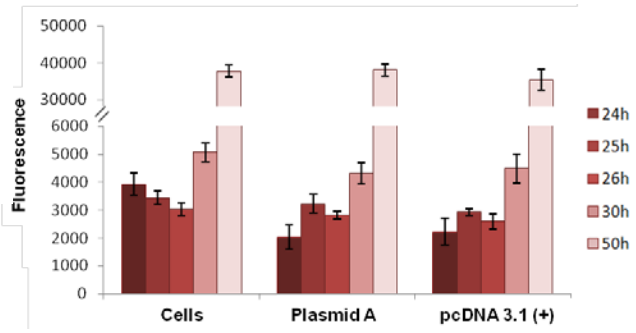


Figure 12. Plasmid A *in vitro* testing. Hepa 1-6 cells were transfected with Plasmid A and cell viability assessed by alamarBlue® at 24, 25, 26, 30 and 50h p.i. No differences are observed between the different conditions at the given time points.

CONCLUSIONS AND FUTURE PERSPECTIVES

Since early as the 1970's, it is known that immunization of humans with *Plasmodium falciparum* sporozoites, attenuated by radiation (*Pf*RAS), is able to induce and establish a long-lasting and completely sterile immune response against further infection with infectious *P. falciparum* sporozoites (Clyde *et al*., 1973). Presently, and after decades of deep research, a vaccine based on such principle is being tested in phase I clinical trials (Vaughan *et al*., 2010). However, the exact mechanisms by which such strong and efficient immune responses are triggered remain unknown. In the last years, observations that cells infected with attenuated parasites undergo apoptosis (Leirião *et al*., 2005b; van Dijk *et al*., 2005) prompted the elaboration of the hypothesis that this event is the player in the induction of the immune response: apoptosis of infected cells leads to the formation of apoptotic bodies filled with parasite antigens. These apoptotic bodies can be therefore phagocytosed by antigen presenting cells that will, afterwards, present these exogenous antigens to the immune system and thus, induce the immune responses responsible for the complete protection against further infections.

The present work purposed precisely to elucidate whereas apoptosis of cells infected with attenuated parasites is involved in the generation of the effective immune protection, triggered upon immunization with these parasites. The results herein presented appear to support the proposed hypothesis: livers of infected mice with *P. berghei* radiation attenuated sporozoites (RAS) present a higher level of apoptosis, and the abrogation, or at least the decrease, of caspase-3 mediated apoptosis in the livers of C57BL/6 caspase-3 deficient mice results in the partial establishment of protective immunity by attenuated sporozoites. Although it was not possible to completely exclude that the acquisition of partial immunity was due to a deficiency in the immune system of the used mice, it appears that the observed phenotype is due to the reduced level of apoptosis in these mice livers, instead of a deficiency in their immune system. Therefore, in the near future, another chimeric mice immunization experiment will be performed, with more mice per group. Moreover, other immunization experiments, with more Casp3KO mice per group (from the same age range as the mice used in the chimeric experiment) will be also performed, in order to elucidate and establish the phenotype observed in the immunized Casp3KO mice.

To further test the involvement of apoptosis of infected cells, a transgenic *P. berghei* ANKA parasite, capable of inducing host cell apoptosis during its pre-erythrocytic stages was idealized. We perspective that this parasite, which is not yet completely constructed, will be able to induce protection against further infections, similarly to the one induced by immunization with attenuated sporozoites. Moreover, we thought of performing more fundamental assays, for instance, to set up the time course of persistence of attenuated sporozoites inside the host cell and when do they start to be unable to prevent host cell to undergo apoptosis both in C57BL/6 and Casp3KO mice primary hepatocytes. After being elucidated the time point p.i. where apoptosis of RAS-infected cells is at maximum, other assays aiming to observe apoptosis of RAS-infected hepatocytes *in vivo* and the presence or not of antigen presenting cells, both in C57BL/6 and Casp3KO mice, will be performed, such the TUNEL assay in liver histological sections.

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